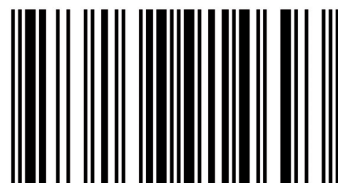# Animal Cell Culture Technology

Animal cell culture has become an essential tool for the study of most biochemical and physiological processes and the use of large-scale animal cell culture has become increasingly important to the commercial production of specific compounds for the pharmaceutical industry. Reflecting the latest developments and trends in the field, the new topics include the latest theory of the biological clock of cell lines, the development of improved serum-free media formulations, the increased understanding of the importance and control of protein expression, and the humanization of antibodies for therapeutic use and also the applications in sericulture and allied fields.

A Biotechnology professional with a Doctor of Philosophy in Biotechnology (PhD) & Master of Philosophy (MPhil) in Biotechnology; possess 15+ years of experience across the industry. Possess a strong and consistently good academic background with work exposure in the area of Biotechnology.

Animal Cell Culture Technology

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Principles and Applications

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Chapter 1

Introduction to Animal cell culture technology

1. Introduction

Culturing of animal cells outside the tissue (*in vitro*) from which they were obtained is the process of animal cell culture. Animal cell culturing is carried out under strict laboratory conditions of asepsis, sterility and controlled environment involving physico-chemical parameters mimicking the *in vivo* environment, so that the cells shall survive and proliferate in a controlled manner. Theoretically, cells of any type can be cultured upon procurement in a viable state from any organ or tissue. However, all types of cells are not capable of survival in such an artificial environment (media) because of many reasons on which the artificial environment may fail to mimic the biochemical parameters of the source environment. These parametes include the absence of growth regulators, cell to cell signal molecules, etc. An artificial environment (media) comprises an appropriate source of energy for the cells which they can easily utilize and compounds which regulate the cell cycle. A typical media may or may not contain serum. Both types of media have their own sets of advantages and disadvantages. Under optimal conditions of maintenance, the cell culture established can be sub-cultured (passaging) until a pure-culture of specific cell type is obtained. This should be repeated to maintain as a cell-line. As a matter of fact, cell lines from cancerous tissues have also been established. The presence of excess growth regulators or other factors may often render the cells to undergo rapid uncontrolled proliferation resulting in a cancerous state. Good examples of established cell lines are HeLa, BHK, Vero, CHO etc. Animal cell culture can be explored a potential tool in drug testing prior to its usage on animals, production of therapeutically significant biological compounds like hormones and proteins on an industrial scale has been made simpler, faster and more efficient by the use of cell lines in the place of the living animal themselves, production of vaccines against many viral infections and diseases, studies on regenerative medicine, active research on stem cell culture, proliferation leading to organogenesis. Animal cell culture also finds application in the preservation of highly valuable cord blood cells which are nothing but stem cells specific to an individual and also in assisted conception.

The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a common laboratory technique in the mid-1900s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century.

The 19th-century English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body. In 1885, Wilhelm

Roux removed a portion of the medullary plate of an embronic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale University, published results of his experiments from 1907-1910, establishing the methodology of tissue culture.

Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The Salk polio vaccine was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Welter and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures.

2. Laboratory equipment in animal cell culture technology

2.1 Laboratory Design

The most under-rated aspect of cell and tissue culture is the need to design the facility to ensure that a good quality material is produced in a safe and efficient manner. Tissue culture is undertaken in laboratories that have been adapted for the purpose and in conditions that are not ideal. However, since a few basic guidelines are adopted this should not compromise the work.

There are several aspects to the design of a good tissue culture facility. Ideally work should be conducted in a single use facility which, if at all possible should be separated into an area reserved for handling newly received material (quarantine area) and an area for material which is known to be free of contaminants (main tissue culture facility). If this is not possible work should be separated by time with all manipulations on clean material being completed before manipulations involving the 'quarantine material'. Different incubators should also be designated. The work surfaces should be thoroughly cleaned between activities. All new material should be handled as 'quarantine material' until it has been shown to be free of different microbial contaminants (particularly mycoplasma). Performing tissue culture in a shared facility requires considerable planning and it is essential that a good technique is used throughout to minimize the risk of microbial contamination.

For most cell lines, the laboratory should be designated to at least Category 2 based on the Advisory Committee on Dangerous Pathogens (ACDP) guidelines (ACDP, 1985)*. However, the precise category required is dependent upon the cell line and the nature of the work proposed. The guidelines make recommendations regarding the laboratory environment including lighting, heating, the type of work surfaces and flooring and provision of hand wash facilities. In addition, it is recommended that laboratories should be run at air pressures that are negative to corridors to contain any risks within the laboratory.

2.2 Microbiological Safety Cabinets

A microbiological safety cabinet is probably the most important piece of equipment because when operated correctly, it will provide a clean working environment for the product, whilst protecting the operator from aerosols. In these cabinets operator and/or product protection is provided through the use of HEPA (high efficiency particulate air) filters. The level of containment provided varies according to the class of cabinet used.

Cabinets may be ducted to atmosphere or re-circulated through a second HEPA filter before passing to atmosphere.
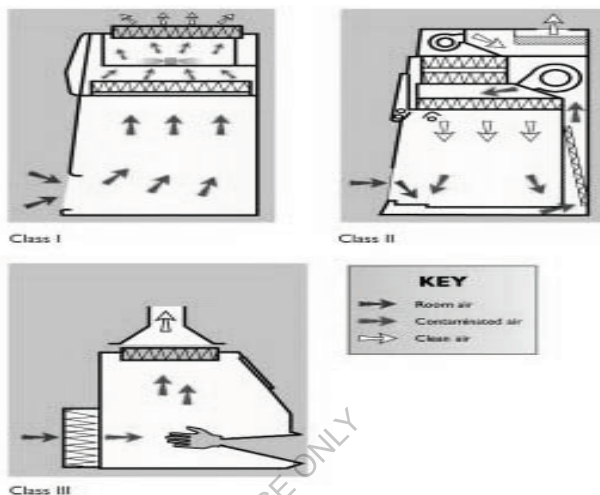
Figure 1. Diagram of microbiological safety cabinet airflow patterns

Environmental monitoring with Tryptose Soya Broth agar settle plates inside the cabinet for a minimum of four hours should be a good indicator of how clean a cabinet is. There should be no bacterial or fungal growth on such plates.

In most cases a class II cabinet is adequate for animal cell culture. However, each study must be assessed for its hazard risk and it is possible that additional factors, such as a known virus infection or an uncertain provenance, may require a higher level of containment.

2.3 Centrifuges

Centrifuges are used routinely in tissue culture as part of the subculture routine for most cell lines and for the preparation of cells for cryopreservation. By their very nature centrifuges produce aerosols and thus it is necessary to minimize this risk. This can be achieved by purchasing models that have sealed buckets. Ideally the centrifuge should have a clear lid so that the condition of the load can be observed without opening the lid. This will reduce the risk of the operator being exposed to hazardous material if a centrifuge tube has broken during centrifugation. Care should always be taken not to over-fill the tubes and to balance them carefully. These simple steps will reduce the risk of aerosols being generated. The centrifuge should be situated where it can be easily accessed for cleaning and maintenance. Centrifuges should be checked frequently for signs of corrosion.

2.4 Incubators

Cell cultures require a strictly controlled environment in which to grow. Specialist incubators are used routinely to provide the correct growth conditions, such as temperature, degree of humidity and CO_2 levels in a controlled and stable manner. Generally they can be set to run at temperatures in the range 28ºC (for insect cell lines) to 37ºC (for mammalian cell lines) and set to provide CO_2 at the required level (e.g., 5-10%). Some incubators also have the facility to control the O_2 levels. Copper-coated incubators are also now available. These are reported to reduce the risk of microbial contamination within the incubator due to the microbial inhibitory activity of copper. The inclusion of water bath treatment fluid in the incubator water trays will also reduce the risk of bacterial and fungal growth in the water trays. However, there is no substitute for regular cleaning **(Figure. 2).**
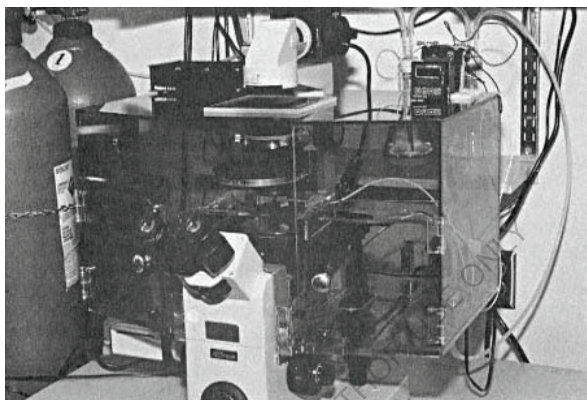
Figure. 2 An incubator box can be constructed to fit over the entire microscope and provide temperature and CO_2 control for time-lapse microcinematography.

2.5 Work Surfaces and Flooring

To maintain a clean working environment the laboratory surfaces including benchtops, walls and flooring should be smooth and easy to clean. They should also be waterproof and resistant to a variety of chemicals (such as acids, alkalis, solvents and disinfectants). In areas used for the storage of materials in liquid nitrogen, the floors should be resistant to cracking if any liquid nitrogen is spilt. Refer to Section 7.3 for safety considerations on the use of liquid nitrogen. In addition, the floors and walls should be continuous with a covered skirting area to make cleaning easier and reduce the potential for dust to accumulate. Windows should be sealed. Work surfaces should be positioned at a comfortable working height (Figure. 3).
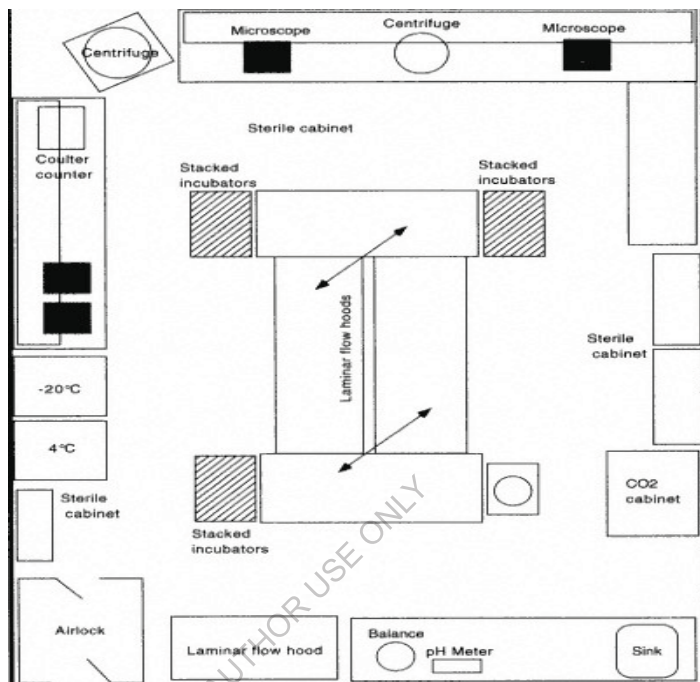
Figure. 3 Suggested floor plan for a standard tissue culture facility

2.6 Plastic ware and Consumables

Almost every type of cell culture vessel, together with support consumables such as tubes and pipettes, are commercially available as a single use, sterile packed, plastic ware. The use of such plastic ware is more cost effective than recycling glassware, enables a higher level of quality assurance and removes the need for validation of cleaning and sterilization procedures. Plastic tissue culture flasks are usually treated to provide a hydrophilic surface to facilitate attachment of anchorage dependent cells.

2.7 Care and Maintenance of Laboratory Areas

To maintain a clean and safe working environment tidiness and cleanliness are key. Obviously all spills should be dealt with immediately. Routine cleaning should also be undertaken involving the cleaning of all work surfaces both inside and outside of the microbiological safety cabinet, the floors and all other pieces of equipment e.g., centrifuges. Humidified incubators are a particular area for concern due to the potential for fungal and bacterial growth in the water trays. This will create a contamination risk that can only be avoided by regular cleaning of the incubator. All major pieces of equipment should be regularly maintained and serviced by qualified engineers.

For instance

- Microbiological safety cabinets should be checked once in every 6 months to ensure that they are safe to use in terms of product and user protection. These tests confirm that the airflow is correct and that the HEPA filters are functioning properly.
- The temperature of an incubator should be regularly checked with a NAMAS (National Accreditation of Measurement and Sampling, UK), or equivalent calibrated thermometer and the temperature adjusted as necessary.
- Incubator CO_2 and O_2 levels should also be regularly checked to ensure the levels are being correctly maintained.

3 Safety Aspects of Cell Culture and Biosafety levels

3.1 Risk Assessment

The main aim of risk assessment is to prevent injury, protect property and avoid harm to individuals and the environment. The performance of risk assessment is a legal requirement under the Health and Safety at Work Act, UK. There are other EC directives covering Health and Safety at Work, you can visit the European Agency for Safety and Health at Work website www.europe.osha.eu.int for information on legislation and standards, or you should contact your on-site representative. Consequently risk assessments must be undertaken before starting any activity. The assessment consists of two elements:

1. Identifying and evaluating the risks.
2. Defining ways of minimizing or avoiding the risk.

For animal cell culture the level of risk is dependent upon the cell line to be used and is based on whether the cell line is likely to cause harm to humans. The different classifications are given below:

Low risk	Non human/non primate continuous cell lines and some well characterized human diploid lines of finite lifespan (e.g., MRC-5).
Medium risk	Poorly characterized mammalian cell lines.
High risk	Cell lines derived from human/primate tissue or blood.
	Cell lines with endogenous pathogens (the precise categorization is dependent upon the pathogen) – refer to ACDP guidelines, 1985, for details.
	Cell lines used following experimental infection where the categorization is dependent upon the infecting agent.

A culture collection, such as ECACC will recommend a minimum the containment level required for a given cell line based upon its risk assessment. For most cell lines the appropriate level of containment is Category 2. However, this may need to be increased to Category 3 depending upon the type of manipulations to be carried out and whether large culture volumes are envisaged. For cell lines derived from patients with HIV or HTLV Category 3 containment is required.

Containment is the most obvious means of reducing risk. Other less obvious measures include restricting the movement of staff and equipment in and out of the laboratories. A good laboratory practice and good bench techniques such as ensuring work areas are uncluttered, reagents are correctly labeled and stored, are also important for reducing risk and making the laboratory a safe environment in which to work. Staff training and the use of written standard operating procedures and risk assessments will also reduce the potential for harm. Training courses covering the basics of tissue culture safety are offered by ECACC.

3.2 Disinfection

Methods designed for the disinfection/decontamination of culture waste, work surfaces and equipment represent important means for minimizing the risk of harm.

The major disinfectants fall into four groups and their relative merits can be summarized as follows:

Hypochlorites (e.g., Chloros, Presept)

- A good general purpose disinfectant
- Active against viruses
- Corrosive against metals and therefore should not be used on metal surfaces e.g., centrifuges
- Readily inactivated by organic matter and therefore should be made fresh daily
- Should be used at 1000 ppm for general use surface disinfection, 2500 ppm in discard waste pots for washing pipettes, and 10,000 ppm for tissue culture waste and spillage

NOTE: When fumigating a cabinet or room using formaldehyde all the hypochlorites must first be removed as the two chemicals react together to produce carcinogenic products.

Phenolics (e.g., Sudol, Hycolin)

- Not active against viruses
- Remains active in the presence of organic matter

Alcohol (e.g., ethanol, isopropanol)

- Effective concentrations 70% for ethanol, 60-70% for isopropanol
- Their mode of activity is by dehydration and fixation
- Effective against bacteria. Ethanol is effective against most viruses but not nonenveloped viruses
- Isopropanol is not effective against viruses

Aldehydes (e.g., glutaraldehyde, formaldehyde)

- Aldehydes are irritants and their use should be limited due to problems of sensitization
- Glutaraldehyde may be used in situations where the use of hypochlorites is not suitable e.g., cleaning of centrifuge bowls or materials constructed of stainless steel that may be attacked or corroded by using hypochlorite solutions.

3.3 Waste Disposal

Any employer has a 'duty of care' to dispose of all biological waste safely in accordance with national legislative requirements. Given below is a list of ways in which tissue culture waste can be decontaminated and disposed of safely. One of the most important aspects of the management of all laboratory-generated waste is to dispose of waste regularly and not to allow the amounts to build up. The best approach is 'little and often'. Different forms of waste require different treatment.

- **Tissue culture waste** (culture medium) - Inactivate overnight in a solution of hypochlorite (10,000 ppm) before disposal to drain with an excess of water
- **Contaminated pipettes** should be placed in hypochlorite solution (2500 ppm) overnight before disposal by autoclaving and incineration
- **Solid waste** such as flasks, centrifuge tubes, contaminated gloves, tissues etc. should be placed inside heavy duty sacks for contaminated waste and autoclaved before incineration.

If at all possible waste should be incinerated rather than autoclaved.

3.4 Biosafety level

From the earliest days of microbiological research, it was recognized that acquiring infections from the agents manipulated was an occupational hazard. The most commonly acquired laboratory infections were caused by bacterial agents; as microbiologists learned to culture animal viruses, they also found ways to become infected with these agents. Significant number of these infections was fatal and that most infections were of unknown origin. Exposure to infectious aerosols was implicated in about 80% reported infections.

Guidelines evolved as a means of protecting microbiological workers based on these data and an understanding of the risks associated with various manipulations of many agents transmissible by different routes. These guidelines work from the premise that safe work sites result from a combination of engineering controls, management policies, work practices and procedures, and, occasionally, medical interventions. The different biosafety levels developed for microbiological and biomedical laboratories provide increasing levels of personnel and environmental protection.

There is a definite hierarchy of administrative controls that need to be in effect. Upper level management must set the general tone that safety is a high priority at their institute. Although this is often expressed in broad policy statements, it must be supported by resource allocation decisions: financial, personnel staffing, training, a safety performance reward structure, etc. For each biosafety level there are also specific supervisory qualifications as assurance that the laboratorians are provided appropriate role models and knowledgeable mentors. Crucial to safe working conditions are various types of specialized equipment available to serve as primary barriers between the microorganism and the laboratorian. These range from simple gloves and other personnel protective equipment to simple (sealed centrifuge heads) or complex (biosafety cabinets) containment devices.

A biosafety level is the level of the biocontainment precautions required to isolate dangerous biological agents in an enclosed facility. The levels of containment range from the lowest biosafety level 1 to the highest at level 4. In the United States, the Centres for Disease

Contol and Prevention (CDC) have specified these levels. In the European Union, the same biosafety levels are defined in a directive.

3.4.1 Levels
3.4.1.1 Biosafety Level 1

BSL-1 is appropriate for working with microorganisms that are not known to cause disease in healthy humans. This is the type of laboratory found in municipal water-testing laboratories, in high schools, and in some community colleges teaching introductory microbiology classes, where the agents are not considered hazardous. The lay-out of a typical BSL-1 laboratory is shown in.

There is a door that can be closed to keep visitors out of the lab while work with the agents is in progress. Hazard warning signs may be posted on the door indicating any hazards that may be present, including radioactive materials, laser lights, high noise emitting equipment, or toxic chemicals. There is a hand-washing sink available, preferably near the door. Waste materials are segregated according to hazard type, and there is an appropriate chemical decon tray for collecting contaminated implements. Work is done on the open bench, and plastic-backed absorbent pads can be placed on the work surface to collect splatter or droplets associated with the work. The bench tops should be impervious to acid and all furniture should be sturdy. If the windows can be opened in the laboratory, they should be fitted with screens.

The lab should be constructed in such a manner that it can be easily cleaned and decontaminated. At BSL-1 there is no specific recommendation that the laboratory be isolated from other parts of the building. Although there is no specific biological safety reason for having more than six air changes per hour in a BSL-1 laboratory, it may be necessary if there are volatile or toxic chemicals in use. In general, inward directional airflow is the ideal.

At BSL-1, standard microbiological practices include the use of mechanical pipetting devices, having a prohibition on eating, drinking and smoking in the lab, and requiring hand washing by all persons when they finish their work or when exiting the laboratory. Persons working in the lab should wear a lab coat to protect their street clothes. It is a recommended practice to wear gloves while manipulating the agents. Additional protective equipment may include working behind a splatter shield or wearing eye or face protection. At BSL-1, no special precautions are needed.

Hand washing is one of the most important procedures that can be used by laboratorians to prevent removal of unwanted microbiological agents, radioactive materials, or chemicals from the laboratory environment. Use of liquid soap is generally preferable to bar soap; twenty seconds of vigorous lathering will remove most of these materials very effectively. After drying your hands with a paper towel, you can use the towel to turn off the faucets and thus prevent recontaminating your hands.

The scientist who provides overall supervision to a BSL-1 laboratory needs to have general training in microbiology or a related science. The supervisor is responsible for establishing the general lab safety procedures and for ensuring that each laboratorian is properly educated in these procedures. Lab personnel, conversely, need to accept such training and follow the proscribed protocols.

3.4.1.2 Biosafety Level 2

The facility, the containment devices, the administrative controls, and the practices and procedures that constitute BSL-2 are designed to maximize safe working conditions for laboratorians working with agents of moderate risk to personnel and the environment. The agents manipulated at BSL-2 are often ones to which the workers have had exposure to in the community, often as children, and to which they have already experienced an immune response. Unlike the guidelines for BSL-1, there are a number of immunizations recommended before working with specific agents. Most notable is hepatitis B virus immunization which is recommended by the Occupational Safety and Health Administration for persons, including laboratorians, at high risk of exposure to blood and blood products. These agents are generally transmissible following ingestion, exposure of mucous membranes, or intradermal exposure. Eating, drinking and smoking are prohibited in BSL-2 laboratories, and extreme precautions are taken while handling needles and other sharp instruments.

The basic lay-out of a BSL-2 laboratory is depicted in. Access to the laboratory is restricted by the supervisor, who establishes the biosafety level, the need for specified personal protective equipment, the need for training, or other appropriate requirements. The door to the laboratory is kept closed to minimize unnecessary access by casual visitors, vendors, or persons not needing to be in the laboratory. There is no requirement for directional inward air flow in a BSL-2 laboratory, except as may be required for chemical odor control; however, many BSL-2 laboratories opt for this feature.

Some work may be done on the open bench by persons wearing appropriate protective clothing or gear. Any work that may produce splatters or aerosols of infectious materials should be done inside a biological safety cabinet (BSC) or other containment device, such as aerosol-containing centrifuge cups. Waste materials need to be segregated into chemical, radioactive, bio-hazardous, or general waste streams. Infectious waste should be decontaminated (by treating with chemical disinfectants or by steam autoclaving).

As the biosafety level increases, all those microbiological practices and procedures delineated for the lower level(s) are carried forward to the next higher level. Thus, the standard microbiological practices found at BSL-1 are still in effect at BSL-2, with emphasis on wearing gloves, using mechanical pipetting devices, and attention to handling sharps. In any situation, do not break or bend needles; in most situations it is prudent to use single-use needles and syringes. Do not recap needles. Needles and syringes, butterfly needles and associated tubing, and similar devices should be discarded intact into a puncture- and leak-proof container. Other sharps items (such as broken glass) should not be handled by hand. Consider substituting plastic ware for glass laboratory items.

Specific policies and procedures regarding access to the BSL-2 laboratory should be developed and posted. On the one hand, it is prudent to allow entry to repair technicians or engineers only if they are very familiar with the activities of the lab or are escorted by a laboratorian that is. Conversely, it needs to be emphasized that the posting of a BSL-2 biohazard sign on the door does not mean that the agent is everywhere in the room; rather, the agent is normally confined to the BSC, an incubator or refrigerator or freezer. It is prudent to schedule entry by non-laboratorians to times when there is no active work with the agent being conducted.

A leak proof box, preferably equipped with a gasket seal lid, should be used for transport of infectious materials from one location to another. This is particularly important when moving samples from patient care areas to laboratories, or from an off-site collection centre to the lab.

Storing a base-line serum sample may be required before working in certain laboratories. This sample can be used at to compare with future serum samples to determine any changes in immunological response to the agents used in the laboratory. Alternatively, a base-line serum sample may be drawn at the time of a possible exposure, then compared to a future sample for possible rising antibody titer. In any event, written employee informed consent must be obtained before obtaining the sample and for subsequent testing. Informed consent is also needed for any immunizations that are offered.

Other special practices include: decontaminating work surfaces after completing the work with the infectious materials, keeping non-research animals out of the laboratory, and reporting all spills and accidents. An incident log book is a useful means for recording events that have gone wrong; it is important to document these events, not for punitive action, but to be able to better understand what happened with an eye to preventing similar events in the future.

At BSL-2, all work that might create aerosols of infectious materials should be done in containment. The most common device is the biological safety cabinet, and the most common cabinet in use is referred to as a Class II, type A BSC, shown in cross-sectional diagram in. Room air is drawn in at the face opening and is immediately drawn through the front grille (A) and under the work surface. The air is then blown (F) through the rear air plenum (E) to the top of the cabinet where it is divided into two chambers (D). Thirty percent of the air is exhausted out of the cabinet (C) through a high efficiency particulate air (HEPA) filter into the laboratory room. The remaining seventy per cent of the air (B) is directed through another HEPA filter down onto the work surface in a laminar flow directional air pattern. The typical HEPA filter removes 99.97% of all particles that are 0.3 micron or larger in size, which means that all microbial agents will be trapped in the filter. The air returned to the laboratory and delivered to the work surface is virtually sterile, which means that an open flame (Bunsen burner) is not needed within the BSC.

Before materials are introduced into the BSC, they should be wiped with 70% alcohol to remove any external contaminants. Experience has shown that clean materials should be kept to one side of the work surface, dirty items on the other. Management of workflow within the BSC is crucial to preventing cross-contamination. Rapid air movement outside the cabinet (caused by co-workers walking past, air supply vents directed across the face of the BSC, etc.) will interrupt the rather fragile air curtain, which may cause air-borne contaminants in the cabinet to be drawn into the lap of the worker. The chair should be adjusted so that the lower portion of the sash is even with the worker's armpits.

Any paper or plastic materials introduced into the BSC should not be allowed to interfere with air flow through the front or rear grilles. The downward airflow from the supply filter "splits" about one third of the way into the cabinet; in the front third, air moves to the front grille, with the remainder of the air flowing to the rear. This means that aerosol-generating activities should be performed towards the rear of the cabinet to provide further worker protection.

Infectious waste materials should be chemically disinfected or, preferably, decontaminated in a steam autoclave. Infectious waste materials to be removed from a BSC should be placed in a pan or tray that can be covered during transport to the autoclave, or placed in a biohazard autoclave bag. By placing an inch or two of water in the bag before sealing it by transport, steam will be generated within the bag during the autoclave cycle.

The supervisor of a BSL-2 laboratory should be a competent scientist who has a technical understanding of the risks associated with the microbiological agents in use. The supervisor limits access to those persons who have received the appropriate immunizations and establishes the personal protective standards for the laboratory; he/she is also responsible for developing the lab's biological safety manual. Laboratory personnel should be aware of the potential hazards associated with the work and be proficient in the specified practices and procedures.

3.4.1.3 Biosafety Level 3

BSL-3 is suitable for work with infectious agents which may cause serious or potentially lethal diseases as a result of exposure by the inhalation route. BSL-3 laboratories should be located away from high-traffic areas. Examples of agents that should be manipulated at BSL-3 are *M. tuberculosis* (research activities), St. Louis encephalitis virus, and *Coxiella burnetii*.

There are some specific secondary barriers needed at BSL-3, that tend to set these laboratories apart from BSL-2. At CDC the current main BSL-3 laboratories are located in a unique high containment building that also houses the BSL-4 laboratory. A typical BSL-3 laboratory lay out is shown in. These laboratories are characterized by having a double-door entry (shown here as an ante-room; other configurations are also used). Because the agents manipulated at BSL-3 are transmissible by the aerosol route, particular attention is given to air movement in these labs. Air moves from areas of lesser contamination to areas of higher contamination, such as from the corridor into the laboratory. Air movement is also single pass; exhaust air is not recirculated to other rooms. Exhaust air does not have to be HEPA filtered, unless local conditions are such that reentrainment into building air supply systems is unavoidable.

All work that may create aerosols or splatter is done inside a biological safety cabinet. Wall, ceiling and floor penetrations are sealed to keep aerosols in and to keep gaseous decontaminants in. The floor is monolithic, and there are continuous cove moldings that extend at least 4" up the wall. Acoustic tiles are not used in BSL-3 laboratories; ceilings should be waterproof for ease of cleaning Centrifuge tubes are placed into containment cups or heads in the BSC, transferred to the centrifuge, spun, then returned to the BSC to be unloaded. In some laboratories the centrifuges themselves are enclosed in a vented area to minimize possible aerosol exposures created in the event of a centrifuge failure. Vacuum lines are protected with HEPA filters so that maintenance personnel are not exposed to infectious aerosols.

Standard microbiological practices are the same as for BSL-1 and BSL-2 laboratories. Class II type A biological safety cabinets are suitable in BSL-3 laboratories. Sometimes Class II type B3 cabinets are installed, requiring thimble connection to the building exhaust systems. Depending on the nature of the work being done in the BSL-3 laboratory, additional personnel protective devices may be worn, such as respirators. When pulmonary protection is

required, the laboratorians need to have appropriate medical evaluations and be trained in proper fit testing and care of their respirators.

Supervisors of BSL-3 laboratories should be competent scientists experienced in working with the agents. They establish criteria for entry into the laboratory, restrict access, develop appropriate practices and procedures, and train the laboratorians. They are also responsible for developing the laboratory safety manual. The lab personnel must rigorously follow the established guidelines, demonstrate proficiency in performing their various procedures, and receive appropriate training. They must participate in specified medical surveillance programs, and report all incidents that constitute potential exposures.

3.4.1.4 Biosafety Level 4

This level is required for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections, agents which cause severe to fatal disease in humans for which vaccines or other treatments are *not* available, such as Bolivian and Argentine hemorrhagic fevers, Marburg virus, Ebola virus, Lassa fever, Crimean-Congonhemorrhagic fever, and other various hemorrhagic diseases. When dealing with biological hazards at this level the use of a Hazmat suit and a self-contained oxygen supply is mandatory. The entrance and exit of a Level Four biolab will contain multiple showers, a vacuum room, an ultraviolet light room, and other safety precautions designed to destroy all traces of the biohazard. Multiple airlocks are used and are electronically secured to prevent both doors opening at the same time. All air and water service going to and coming from a biosafety level 4 lab will undergo similar decontamination procedures to eliminate the possibility of an accidental release.

Agents with a close or identical antigenic relationship to Biosafety Level 4 agents are handled at this level until sufficient data is obtained either to confirm continued work at this level, or to work with them at a lower level.

Members of the laboratory staff have specific and thorough training in handling extremely hazardous infectious agents and they understand the primary and secondary containment functions of the standard and special practices, the containment equipment, and the laboratory design characteristics. They are supervised by qualified scientists who are trained and experienced in working with these agents. Access to the laboratory is strictly controlled by the laboratory director.

The facility is either in a separate building or in a controlled area within a building, which is completely isolated from all other areas of the building. A specific facility operations manual is prepared or adopted. Building protocols for preventing contamination often use negatively pressurized facilities, which, if compromised, would severely inhibit the containment of an outbreak of aerosol pathogens.

Within work areas of the facility, all activities are confined to Class III biological safety cabinets, or Class II biological safety cabinets used with one-piece positive pressure personnel suits ventilated by a life support system. The Biosafety Level 4 laboratory has special engineering and design features to prevent microorganisms from being disseminated into the environment. The laboratory is kept at negative air pressure, so that air flows into the room if the barrier is penetrated or breached. Furthermore, an airlock is used during personnel entry and exit.

The guidelines presented in the CDC/NIH publication *Biosafety in Microbiological and Biomedical Laboratories* present standard and special practices, safety equipment recommendations, and performance standards for the facilities that, taken together, should be considered as optimal for most laboratory situations. There may be instances where unique needs, unknown hazards associated with unknown pathogens, or other contributing requirements will cause supervisors or biosafety professionals to seek higher biosafety requirements. These can be established after appropriate risk assessments have been conducted.

Table 2. List of Biosafety levels

Name	Location	Level	Description
Virology Laboratory of the Queensland Department of Health	Australia, Queensland, Coopers Plains	4	
Australian Animal Health Laboratory	Australia, Victoria, Geelong	4	
Infectious Diseases Unit, St.John's Research Institute	India, Bangalore	3	
All India Institute of Medical Sciences	India, New Delhi	1	One of the premier research institute & conducts studies on major pathogenic organisms. Has been contributed in discovering novel strains & vaccines.
National JALMA Institute for Leprosy & Other Mycobacterial Diseases (NCJILOMD), Agra	India, Agra	3	This facility deals with the Mycobacterial strains & their pathogenicity & epidemiology.
National High Security Laboratory	Australia, Victoria, North Melbourne	4	National High Security Laboratory Operates under the auspice of the Victoria Infectious Diseases Reference Laboratory.
Curtin University of Technology	Australia, Western Australia, Bentley	3	
Fundação Oswaldo Cruz	Brazil, Rio de Janeiro	3	It is unclear whether this facility operates as a BSL-4. Brazil doesn't have any BSL-4 facilities.
University of São Paulo	Brazil, São Paulo	3	It is unclear whether this facility operates as a BSL-4. Brazil doesn't have any BSL-4 facilities.
Instituto Adolf Lutz	Brazil	3	
Instituto Butantan	Brazil	3	
British Columbia Centre for Disease Control	Canada, British Columbia	3	The British Columbia Centre for Disease Control, operates three biosafety level 3 labs.
National Microbiology Laboratory	Canada, Manitoba, Winnipeg	4	Located at the Canadian Science Centre for Human and Animal Health, it is jointly operated by the Public Health Agency of Canada and the Canadian Food Inspection Agency.
Centre National de Biologie Expérimentale	Canada, Quebec, Laval	3	Located at the Institut national de la recherche scientifique.

National Institute for Health and Welfare	Finland, Tilkanmäki, Helsinki	3	Document from National Institute for Health and Welfare that mentios the facility.
Laboratoire P4 Jean Mérieux	France, Rhône-Alpes, Lyon	4	Jean Mérieux laboratory is a co-operation between the Pasteur Institute and INSERM. Note that in France, it is P4 for Pathogen or Protection level 4.
Centre International de Recherches Médicales de Franceville	Gabon	4	This facility is operated by a research organization supported by the French government, operates West Africa's only BSL-4 lab.
Robert Koch Institute	Germany, Berlin	4	The facility was licenced for construction by City of Berlin on November 30, 2008.
Bernhard Nocht Institute for Tropical Medicine	Germany, Hamburg	4	
Philipps University of Marburg	Germany, Marburg	4	The facility is licenced to work with genetically modified organisms
Friedrich Loeffler Institute on Island Riems	Germany, Island Riems	4	Deals especially with virology
Evangelismos	Greece, Athens	3	
Crete University, Pagne hospital, Clinical bacteriology lab	Greece, Heraklion	3	
High Security Animal Disease Laboratory (HSADL)	India, Bhopal	4	This facility deals especially to zoonotic organisms and emerging infectious disease threats.
Institute of Tropical Disease (ITD)	Indonesia, East Java, Surabaya	3	Operated by Institute of Tropical Disease - Airlangga University, Build Cooperation with Japan.
Azienda Ospedaliera Ospedale Luigi Sacco	Italy, Lombardy, Milano	4	A university hospital in Polo Universitario; it contains two special vehicles for transporting infectious persons.
Istituto Nazionale Malattie Infettive	Italy, Rome, Rome	4	This facility, (trans.) National Institute of Infectious Diseases, operates within the Lazzaro Spallanzani Hospital.
Institute for Medical Research (IMR), Ministry of Health	Malaysia, Kuala Lumpur	3	This facility is able to conduct research and tests on acarology, bacteriology, medical entomology, parasitology and virology.
National Institute for Infectious Diseases	Japan, Kantô, Tokyo	3	Located at National Institute for Infectious Diseases, Department of Virology I; this lab has the potential of operating as a BSL-4, however it is limited to perform work on only BSL-3 agents due to opposition from local residents and communities.
Institute of Physical and Chemical Research	Japan, Kantô, Tsukuba		This is a non-operating BSL-4 facility.
Netherlands National Institute for Public Health and the Environment (RIVM)	Netherlands, Bilthoven	3	Currently under construction, it is planned to be finished by the end of 2009. It is planned to operate as a BSL 3 and a BSL 4 facility.

Wuhan Institute of Virology of the Chinese Academy of Sciences	People's Republic of China, Hubei, Wuhan	3	Wuhan Institute of Virology already hosts a BSL-3 laboratory. A distinct BSL-4 facility is currently being built based on P4 standards, the original technology for confinement developed by France. It will be the first at level 4 in China, under the direction of Shi Zhengli.
State Research Center of Virology and Biotechnology VECTOR	Russia, Novosibirsk Oblast, Koltsovo	1	It is one of two facilities in the world that officially hold smallpox. The other Russian BSL-4 facilities have been dismantled.
Defence Science Organization (DSO)	Singapore	4	Defence Science Organization goal is to conduct autopsies during a potential deadly epidemic outbreak. Singapore also has a mobile BSL-4 autopsy facility, perhaps the only one of its kind in the world.
National Institute for Communicable Diseases	South Africa, Johannesburg	4	National Institute for Communicable Diseases of Special Pathogens Unit is one of only two BSL-4 facilities in Africa but the only suit laboratory on the continent.
National Public Health Laboratory (NPHL), Ministry of Health	Malaysia, Sungai Buloh, Selangor	3	The NPHL is established to develop laboratory-based surveillance systems for infectious diseases of epidemic and pandemic potential as part of an early warning system and response strategies for outbreaks of infectious diseases.
Swedish Institute for Infectious Disease Control	Sweden, Solna	4	Swedish Institute for Infectious Disease Control is Scandinavia's P4 facility.
Institute of Virology and Immunoprophylaxis (IVI)	Switzerland, Mittelhäusern	4	This facility only deals with animal diseases which do not transmit to humans, and is the only P4 facility where complete isolation suits are not used.
High Containment Laboratory DDPS (SiLab)	Switzerland, Spiez	4	Under construction, it will start operations in 2010. This laboratory will comply with BSL-4 standards.
Preventive Medical Institute of ROC Ministry of National Defense	Republic of China (Taiwan)	4	
Kwen-yang Laboratory Center of Disease Control	Republic of China (Taiwan)	4	Part of the Department of Health Republic of China.
Health Protection Agency's Centre for Infections	United Kingdom, Colindale	4	Located in the Viral Zoonosis unit.
National Institute for Medical Research	United Kingdom, London	4	
Chemical and Biological Defence Establishment	United Kingdom, Porton Down	4	
Centers for Disease Control and Prevention	United States of America, Georgia, Atlanta	4	Currently operates in two buildings. One of two facilities in the world that officially hold smallpox.
George Mason University	United States of America, Virginia,	3	This facility is currently under construction on a 10-acre site adjacent to George Mason

Biomedical Research Laboratory	Manassas		University's Prince William Campus. It is scheduled to be fully operational in the Spring of 2010.
Georgia State University	United States of America, Georgia, Atlanta	4	Is an older design "glovebox" facility.
Integrated Research Facility	United States of America, Maryland, Fort Detrick	4	Under construction. This facility will be operated by National Institute of Allergy and Infectious Diseases (NIAID), it is planned to begin operating at 2009 at the earliest.
National Biodefense Analysis and Countermeasures Center (NBACC)	United States of America, Maryland, Fort Detrick	4	Under construction, it will be operated for the Department of Homeland Security.
National Institutes of Health (NIH)	United States of America, Maryland, Bethesda	3	Located on the NIH Campus, it currently only operates with BSL-3 agents.
US Army Medical Research Institute of Infectious Diseases (USAMRIID)	United States of America, Maryland, Fort Detrick	4	Old building
US Army Medical Research Institute of Infectious Diseases (USAMRIID)	United States of America, Maryland, Fort Detrick	4	New building, currently under design construction
National Emerging Infectious Diseases Laboratory (NEIDL)	United States of America, Massachusetts, Boston	4	Under construction by Boston University, building and staff training complete, waiting for regulatory approval.
NIAID Rocky Mountain Laboratories	United States of America, Montana, Hamilton	4	Under construction, it is planned to begin operation in 2009 at the earliest.
Stony Brook University Centers for Molecular Medicine Center for Infectious Diseases	United States of America, New York, Stony Brook	3	Operated by State University of New York at Stony Brook, a BSL-3 facility studying Borrelia burgdorferi (Lyme Disease), Yersinia pestis (Bubonic plague) and Francisella tularensis (Rabbit fever)
University of Cincinnati	United States of America, Ohio, Cincinnati	3	University of Cincinnati Medical Sciences Building
Battelle Memorial Institute	United States of America, Ohio, West Jefferson	3	
National Biocontainment Facility	United States of America, Texas, Galveston	4	Opened in 2008, facility is operated by the University of Texas Medical Branch.
Shope Laboratory	United States of America, Texas, Galveston	4	Operated by the University of Texas Medical Branch (UTMB).
Southwest Foundation for Biomedical Research	United States of America, Texas, San Antonio	4	The only privately-owned BSL-4 lab in the US.
Division of Consolidated	United States of America, Virginia,	4	This facility is part of the Department of General Services of the Commonwealth of

Laboratory Services	Richmond		Virginia). It is so called "surge" BSL-4 capacity.
National Bio and Agro-Defense Facility (NBAF)	United States of America, Kansas State University, Manhattan, Kansas	4	Facility to be operated by the Department of Homeland Security, and replace the Plum Island Animal Disease Center. Planned to be operational by 2014.
Plum Island Animal Disease Center	United States of America, New York, Plum Island	3	Facility scheduled to be replaced by the National Bio and Agro-Defense Facility. Researches zoonotic pathogens only.
Saint Louis University Doisy Research Building	United States of America, Missouri, Saint Louis	3	Saint Louis University's new center for biomedical research. Monkeypox is the primary BSL-3 agent studied.
University of California, Berkeley	United States of America, California, Berkeley	3	The UC Berkeley BSL3 Facility is currently housed in a single location but will be expanded in 2011 to include a second site (in a building under construction).
University of California, Los Angeles	United States, California, Los Angeles	3	
Instituto Nacional de Tecnologia Agropecuaria, Buenos Aires	Argentina, Buenos Aires, Castelar	3	*Campo agricola*.
Veterinary Research Institute (VRI), Department of Veterinary Services	Malaysia, Ipoh, Perak	3	This facility created a breakthrough in identifying the Nipah virus, which is classified internationally as a BSL-4 agent.

4 Sources of Cell Lines

Large numbers of cell lines look identical. Cell lines with very different origins and biological characteristics typically cannot be separated on grounds of morphology or culture characteristics. Infection or contamination of a cell line with an adventitious virus or mycoplasma may significantly change the characteristics of the cells but again such contamination will be inapparent. Cell lines will also change with time in culture, and to add to all these natural hazards it is all too easy to mis-label or cross-contaminate different cell lines in a busy cell culture laboratory.

The opportunities for inadvertently introducing error into a cell line are limitless and ever present. It is in the nature of the science that, once introduced, an error will be propagated, compounded, consolidated and disseminated.

The integrity and biological characteristics of a cell line have to be actively maintained by a well-organized system of "husbandry" based on systematic cell banking supported by testing regimens in a structured quality assured environment. Such a controlled environment will only prevail in a dedicated professionally organized cell culture laboratory or cell bank. A small research laboratory with a high throughput of short-term research students, a minimum of permanent laboratory staff and no formal quality management program will find it difficult to maintain its cell lines unchanged over many years.

For all these reasons it is strongly recommended that new cell lines should only be acquired from a specialist, reputable culture collection such as ECACC. Moreover, if a laboratory believes it already has a certain cell line in its liquid nitrogen store, the identity and purity of such a cell line should be questioned in the absence of a well-recorded culture history and recent test data. If there is a doubt, it is straightforward and cost effective to replace such cell stocks with authenticated material from a Culture Collection.

When a Cell Culture Collection "accessions" a new cell line it will characterize the cell line using techniques such as isoenzyme analysis and DNA profiling so that the identity of the cell line can subsequently be verified. The Collection will then establish a hierarchy of Master and Working cell banks, cryopreserved in liquid nitrogen, that are demonstrated free from microbial contamination including mycoplasma. Customers are supplied from this authenticated Working Cell Banks (WCB). Replacement WCB's are manufactured from the original Master Cell Bank (MCB) and the new WCB will again be fully tested.

5 Main Types of Cell Culture

5.1 Primary Cultures

Primary cultures are derived directly from excised, normal animal tissue and cultured either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fibroblasts. The preparation of primary cultures is labor intensive and they can be maintained *in vitro* only for a limited period of time. During their relatively limited life span primary cells usually retain many of the differentiated characteristics of the cell *in vivo*.

5.2 Continuous Cultures

Continuous cultures comprised a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approx. 30) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. The fact that such cell lines senesce after approximately thirty cycles of division means it is essential to establish a system of Master and Working banks to maintain such lines for long periods.

Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumor cells. Tumor cell lines are often derived from actual clinical tumors, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in vivo* characteristics.

5.3 Culture Morphology

Morphologically cell cultures take one of two forms, growing either in suspension (as single cells or small free-floating clumps) or as a monolayer that is attached to the tissue culture flask. The form taken by a cell line reflects the tissue from which it was derived e.g., cell lines derived from blood (leukaemia, lymphoma) tend to grow in suspension whereas cells derived from solid tissue (lungs, kidney) tend to grow as monolayers. Attached cell lines can be classified as endothelial such as BAE-1, epithelial such as HeLa, neuronal such as SH-SY5Y or fibroblasts such as MRC-5 and their morphology reflect the area within the tissue of origin.

There are some instances when cell cultures may grow as semi-adherent cells e.g., B95-8 where there appears to be a mixed population of attached and suspension cells. For these cell lines it is essential that both cell types are subcultured to maintain the heterogeneous nature of the culture.

6 The Cell Environment (including types of culture medium)

In general terms cultured cells require a sterile environment and a supply of nutrients for growth. In addition, the culture environment should be stable in terms of pH and temperature. Over the last 30 years various defined basal media types have been developed and are now available commercially. Originally balanced salt solutions were used to maintain contractility of mammalian heart tissue and Tyrode's salt solution was designed for use in work with primary mammalian cells. These have since been modified and enriched with amino acids, vitamins, fatty acids and lipids. Consequently media suitable for supporting the growth of a wide range of cell types are now available. The precise media formulations have often been derived by optimizing the concentrations of every constituent. Examples of the different media and their uses are given in the table below (Table 3).

Table 3. Different types of culture medium and their uses

Media type	Examples	Uses
Balanced salt solutions	Hanks BSS, Earles salts	Form the basis of many complex media
Basal media	MEM	Primary and diploid cultures.
	DMEM	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas.
	GMEM	Glasgows modified MEM was defined for BHK-21 cells
Complex media	RPMI	Originally derived for human leukaemic cells. It supports a wide range of mammalian cells including hybridomas
	Iscoves DMEM	Further enriched modification of DMEM which supports high density growth
	Leibovitz L-15	Designed for CO_2 free environments
	TC 100 ,	Designed for culturing insect cells
	Grace's Insect Medium,	
	Schneider's Insect Medium	
Serum Free Media	CHO	For use in serum free applications.
	Ham F10 and derivatives, Ham F12, DMEM/F12	NOTE: These media must be supplemented with other factors such as insulin, transferrin and epidermal growth factor. These media are usually HEPES buffered

Insect cells	Sf-900 II SFM, SF Insect-Medium-2	Specifically designed for use with Sf9 insect cells

6.1 Basic Constituents of media

- Inorganic salts
- Carbohydrates
- Amino acids
- Vitamins
- Fatty acids and lipids
- Proteins and peptides
- Serum

Each type of constituent performs a specific function as outlined below:

6.2 Inorganic Salts

The inclusion of inorganic salts in media performs several functions. Primarily they help to retain the osmotic balance of the cells and help regulate membrane potential by provision of sodium, potassium and calcium ions. All of these are required in the cell matrix for cell attachment and as enzyme cofactors.

6.3 Buffering Systems

Most cells require pH conditions in the range 7.2 - 7.4 and close control of pH is essential for optimum culture conditions. There are major variations to this optimum. Fibroblasts prefer a higher pH (7.4 - 7.7) whereas, continuous transformed cell lines require more acid conditions pH (7.0 - 7.4). Regulation of pH is particularly important immediately following cell seeding when a new culture is establishing and is usually achieved by one of two buffering systems; (i) a "natural" buffering system where gaseous CO_2 balances with the CO_3 / HCO_3 content of the culture medium and (ii) chemical buffering using a zwitterion called HEPES.

Cultures using natural bicarbonate/CO_2 buffering systems need to be maintained in an atmosphere of 5-10% CO_2 in air usually supplied in a CO_2 incubator. bicarbonate/CO_2 is low cost, non-toxic and provides other chemical benefits to the cells.

HEPES has superior buffering capacity in the pH range 7.2 - 7.4 but is relatively expensive and can be toxic to some cell types at higher concentrations. HEPES buffered cultures do not require a controlled gaseous atmosphere.

Most commercial culture media include phenol red as a pH indicator so that the pH status of the medium is constantly indicated by the color. Usually the culture medium should be changed / replenished if the color turns yellow (acid) or purple (alkali).

6.4 Carbohydrates

The main source of energy is derived from carbohydrates generally in the form of sugars. The major sugars used are glucose and galactose however some media contain maltose or fructose. The concentration of sugar varies from basal media containing 1g/l to 4.5g/l in some more complex media. Media containing the higher concentration of sugars are able to support the growth of a wider range of cell types.

6.5 Vitamins

Serum is an important source of vitamins in cell culture. However, many media are also enriched with vitamins making them consistently more suitable for a wider range of cell lines. Vitamins are precursors for numerous co-factors. Many vitamins especially B group vitamins are necessary for cell growth and proliferation and for some lines the presence of B12 is essential. Some media also have increased levels of vitamins A and E. The vitamins commonly used in media include riboflavin, thiamine and biotin.

6.6 Proteins and Peptides

These are particularly important in serum free media. The most common proteins and peptides include albumin, transferrin, fibronectin and fetuin and are used to replace those normally present through the addition of serum to the medium.

6.7 Fatty Acids and Lipids

Like proteins and peptides these are important in serum free media because they are normally present in serum. For example, cholesterol and steroids essential for specialized cells.

6.8 Trace Elements

These include trace elements such as zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a detoxifier and helps remove oxygen free radicals.

Whilst all media may be made from the basic ingredients this is time consuming and may predispose to contamination. For convenience most media are available as ready mixed powders or as 10x and 1x liquid media. All commonly used media are listed in the Sigma-Aldrich Life Science Catalogue. If powder or 10x media are purchased it is essential that the water used to reconstitute the powder or dilute the concentrated liquid is free from mineral, organic and microbial contaminants. It must also be pyrogen free, water, tissue culture grade). In most cases water prepared by reverse osmosis and resin cartridge purification with a final resistance of 16-18Mx is suitable. Once the media is prepared, it should be filtered and sterilized before use. Obviously purchasing 1x liquid media direct from Sigma eliminates the need for this.

6.9 Serum

Serum is a complex mix of albumins, growth factors and growth inhibitors and is probably one of the most important components of cell culture medium. The most commonly used serum is fetal bovine serum. Other types of serum are available including newborn calf serum and horse serum. The quality, type and concentration of serum can all affect the growth of cells and it is therefore important to screen batches of serum for their ability to support the growth of cells. In addition there are other tests that may be used to aid the selection of a batch of serum including cloning efficiency, plating efficiency and the preservation of cell characteristics.

Serum is also able to increase the buffering capacity of cultures that can be important for slow growing cells or where the seeding density is low (e.g., cell cloning experiments). It also helps to protect against mechanical damage which may occur in stirred cultures or whilst using a cell scraper. A further advantage of serum is the wide range cell types with which it can be used despite the varying requirements of different cultures in terms of growth factors. In addition serum is able to bind and neutralize toxins. However, serum is subject to batch-batch variation that makes standardization of production protocols difficult. There is also a risk of contamination associated with the use of serum. These risks can be minimized by obtaining serum from a reputable source because suppliers of large quantities of serum perform a battery of quality control tests and supply a certificate of analysis with the serum. In particular serum is screened for the presence of bovine viral diarrhea virus (BVDV) and mycoplasma. Heat inactivation of serum (incubation at 56ºC for 30 minutes) can help to reduce the risk of contamination because some viruses are inactivated by this process. However the routine use of heat inactivated serum is not an absolute requirement for cell culture. The use of serum also has a cost implication not only in terms of medium formulation but also in downstream processing. A 10% FBS supplement contributes 4.8 mg of protein per ml of culture fluid, which complicates downstream processing procedures.

6.10 Guidelines for serum use

Fetal bovine serum (FBS) has been used to prepare a number of biological and has an excellent record of safety. The recognition of bovine spongiform encepalopathy (BSE) in 1986 and its subsequent spread into continental Europe alongside the announcement of the probable link between BSE and a new variant of Creutzfeldt Jacob disease in Humans, stimulated an increased concern about safe sourcing of all bovine materials. In 1993, the Food and Drug Administration (FDA) "recommended against the use of bovine derived materials from cattle which have resided in, or originated from countries where BSE has been diagnosed. The current European Union guidelines on viral safety focus on sourcing, testing and paying particular attention to the potential risk of cross contamination during slaughtering or collection of the starting tissue. As far as BSE is concerned, the EU guidelines on minimizing the risk of BSE transmission via medicinal products, CPMP/BWP/877/96, recommends the main measures to be implemented to establish the safety of bovine material versus the BSE risk. Again similarly the focus is on geographical origin, the age of the animals, the breeding and slaughtering conditions, the tissue to be used and the conditions of its processing.

The use of FBS in production processes of medicinal products is acceptable provided good documentation on sourcing, age of the animals and testing for the absence of adventitious agents is submitted. All responsible suppliers of FBS for bio-pharmaceutical applications will provide such documentation.

Recent regulatory requirements in Europe stress the importance of justifying the use of material of bovine, caprine or ovine origin in the production of pharmaceutical products. Although FBS has been used for many years in the production process of many medicinal products such as viral vaccines and recombinant DNA products, at present there is a justified trend to remove all material of animal origin from manufacturing processes. Sigma-Aldrich has recognized this growing trend and works closely with customers to optimize animal free media formulations to meet each customer's cell culture requirements.

Similarly, the FDA has similar guidelines when accepting regulatory submissions. The FDA regulates all medicinal products for Human use, such as therapeutics, vaccines and diagnostics.

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Chapter 2
Basic principles

1 Introduction

Cell culture has become an indispensable technology in many branches of life sciences that provides the basis for studying the regulation of cell proliferation, differentiation and product formation in carefully controlled conditions with processes and analytical tools which are scalable from the level of the single cell to in excess of 10 kg wet weight of cells. Cell culture has also provided the means to define almost the entire human genome and to dissect the pathways of intracellular and intercellular signalling which ultimately regulate gene expression. From its ancestry in developmental biology and pathology, this discipline has now emerged as a tool for molecular geneticists, immunologists, surgeons, bioengineers and manufacturers of pharmaceuticals while still remaining a fundamental tool to the cell biologist, whose input is vital for the continuing development of the technology.

Cell culture has matured from a simple microscope driven observational science to a universally acknowledged technology with roots set as deep in industry as they are in academics. It stands among microelectronics, avionics, astrophysics and nuclear engineering as one of the major bridges between fundamental research and industrial exploitation, and in the current climate, perhaps the more commercial aspects will ensure its development for at least several more decades. The prospects for genetic therapy and tissue replacement are such that the questions are rapidly becoming ethical, as much as technical, as new opportunities arise for genetic manipulation, whole animal cloning and tissue transplantation.

2 Biology of cells in culture
2.1 Origin and characterization

The list of different cell types which can be grown in culture is extensive, includes representatives of most major cell types, and has significantly increased, due largely to the improved availability of selective media and specialized cell cultures through commercial sources such as Clonetics.

The use of markers that are cell type specific has made it possible to determine the lineage from which many of these cultures were derived, although the position of the cells within the lineage is not always clear. During propagation, a precursor cell type will tend to predominate rather than a differentiated cell. Consequently, a cell line may appear to be heterogeneous as some cultures, such as epidermal keratinocytes can contain stem cells, precursor cells and mature differentiated cells. There is constant renewal from the stem cells, proliferation and maturation in the precursor compartment and terminal and irreversible differentiation in the mature compartment. Other cultures such as dermal fibroblasts, contain a relatively uniform population of proliferating cells at low cell densities (about 10^4 cells/cm^2) and an equally uniform, more differentiated, non-proliferating population at high cell densities (10^5 cells/cm^2). This high density population of fibrocyte-like cells can re-enter the cell cycle if the cells are trypsinized or scraped (by making a cut in the monolayer) to reduce the cell density or create a free edge. Most of the cells appear to be capable of proliferation and there is little evidence of renewal from a stem cell compartment. Culture heterogeneity also results from multiple lineages being present in the cell line. The only

unifying factors are the selective conditions of the medium and substrate and the predominance of the cell type (or types) which have the ability to survive and proliferate. This tends to select a common phenotype but due to the interactive nature of growth control may obscure the fact that the population contains several distinct phenotypes only detectable by cloning.

Nutritional factors like serum, Ca^{2+} ions, hormones, cell and matrix interactions and culture density can affect differentiation and cell proliferation. Hence, it is not only essential to define the lineage of cells being used, but also to characterize and stabilize the stage of differentiation by controlling cell density and the nutritional and hormonal environment to obtain a uniform population of cells which will respond in a reproducible fashion to given signals.

As the dynamic properties of cell culture (proliferation, migration, nutrient utilization, product secretion) are sometimes difficult to control and the complexity of cell interactions found *in vivo* can be difficult to recreate *in vitro,* there have been numerous attempts to either retain the structural integrity of the original tissue using traditional organ culture or to recreate it by combining propagated cells of different lineages in organotypic culture. Among the most successful examples of the latter are the so called skin equivalent models, where epidermal keratinocytes are co-cultured with dermal fibroblasts and collagen in filter well inserts or some similar mechanical support. The result is a synthetic skin suitable for grafting and now being evaluated for tests of irritancy and inflammation.

2.2 Differentiation and Development

As propagation of cell lines requires that the cell number increases, culture conditions have evolved to favour maximal cell proliferation. It is not surprising that these conditions are often not conducive to cell differentiation where cell growth is severely limited or completely abolished. Those conditions which favour cell proliferation are low cell density, low Ca^{2+} concentration (100-600 mM), and the presence of growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). High cell density (> 1 x 10^5 cells/cm^2), high Ca^{2+} concentration (300-1500 mM), and the presence of differentiation inducers, hormones such as hydrocortisone, paracrine factors such as IL-6 and KGF and nerve growth factor retinoids and planar polar compounds, such as dimethyl sulfoxide will favour cytostasis and differentiation. The role of serum in differentiation is complex and depends on the cell type and medium used. A low serum concentration promotes differentiation in oligodendrocytes whereas; a high serum concentration causes squamous differentiation in bronchial epithelium. In the latter case, this is due to transforming growth factor B (TGFB) released from platelets. Because of the undefined composition of serum and the ever-present risk of adventitious agents such as viruses, controlled studies on selective growth and differentiation are best conducted in serum-free media.

The establishment of the correct polarity and cell shape may also be important, particularly in epithelium. Many reports have shown that growing cells to high density on a floating collagen gel allows matrix interaction, access to medium on both sides and particularly, to the basal surface where receptors and nutrient transporters are expressed. The plasticity of the substrate can facilitate a normal cell shape and normal polarity of the interactive ligands in the basement membrane. Different conditions may be required for propagation and differentiation and hence an experimental protocol may require a growth

phase for expansion and to allow for replicate samples followed by a non-growth maturation phase to allow for increased expression of differentiated functions.

Mammalian cell lines command an effective monopoly for the production of therapeutic proteins that require post-translational modifications. This unique advantage outweighs the costs associated with mammalian cell culture, which are far grater in terms of development time and manufacturing when compared to microbial culture. The development of cell lines has undergone several advances over the years, essentially to meet the requirement to cut the time and costs associated with using such a complex hosts as production platforms.

Development of cell lines and the cell engineering approach can be used to enhance productivity, improve cell function, glycosylation and secretion and control apoptosis. Expression engineering including epigenetics and the use of technologies to overcome positional dependent inactivation, the use of promoter and enhancer sequences for expression of various transgenes, site directed engineering of defined chromosomal sites, and examination of the role of eukaryotic nucleus as the controller of expression of genes that are introduced for production of a desired product. It includes a review of selection methods for high producers and an application developed by a major biopharmaceutical industry to expedite the cell line development process. The potential of cell engineering approch to enhance cell lines through the manipulation of genes that plays important roles in key metabolic and regulatory pathways.

3 Choice of materials
3.1 Cell type

The cell type chosen will depend on the question being asked. For some processes, such as DNA synthesis, response to cytotoxins or apoptosis, the cell type may not matter, provided the cells are competent. In other cases a specific process will require a particular cell type, for example surfactant synthesis in the lung will require a fresh isolate of type II pneumocytes or a cell line such as NCI-H441, which still expresses surfactant proteins. A reasonable first step would be to determine from the literature whether a cell line exists with the required properties.

3.2 Source of tissue
3.2.1 Embryo or adult?

Cultures derived from embryonic tissues survive and proliferate better than those from the adult in general. This presumably reflects the lower level of specialization and higher proliferative potential in the embryo. Adult tissues usually have a lower growth fraction and a higher proportion of non-replicating specialized cells, often within a more structured and less readily disaggregated extracellular matrix. Initiation and propagation are more difficult, and the lifespan of the culture often shorter. Embryonic or fetal tissue has many practical advantages but it must be remembered that in some instances the cells will be different from adult cells and it cannot be assumed that they will mature into adult-type cells unless this can be confirmed by appropriate characterization.

Examples of widely used embryonic cell lines are the various 3T3 lines (primitive mouse mesodermal cells) and WI-38, MRC-5, and other human fetal lung fibroblasts. Mesodermally derived cells (fibroblasts, endothelium and myoblasts) are on the whole easier

to culture than epithelium, neurons or endocrine tissue. This selectivity may reflect the extensive use of fibroblast cultures during the early development of culture media and the response of mesodermally derived cells to mitogenic factors present in serum. Selective media have now been designed for epithelial and other cell types, and with some of these it has been shown that serum is inhibitory to growth and may promote differentiation. Primary culture of epithelial tissues such as skin, lung, and mammary gland is routine in some laboratories and prepared cultures are available commercially.

3.2.2 Normal or neoplastic?

Normal tissue usually gives rise to cultures with a finite lifespan, while cultures from tumours can give rise to continuous cell lines. However, there are several examples of continuous cell lines (BHK-21 hamster kidney fibroblasts, MDCK dog kidney epithelium, 3T3 fibroblasts) which have been derived from normal tissues and which are non-tumorigenic. Normal cells generally grow as an undifferentiated stem cell or precursor cell and the onset of differentiation is accompanied by a cessation in cell proliferation which may be permanent. Some normal cells such as fibrocytes or endothelium are able to differentiate and still de-differentiate and resume proliferation and in turn re-differentiate while others, such as squamous epithelium, skeletal muscle, and neurons, once committed to differentiate, appear to be incapable of resuming proliferation. Cells cultured from neoplasms such as B16 mouse melanoma, can express at least partial differentiation, while retaining the capacity to divide. Many studies of differentiation have taken advantage of this fact and used differentiated tumours such as hepatomas and human and rodent neuroblastomas although whether this differentiation is normal are not known.

Tumour cells can often be propagated in the syngeneic host providing a cheap and simple method of producing large numbers of cells, albeit with lower purity. Where the natural host is not available tumours can also be propagated in animals with a compromised immune system, with greater difficulty and cost but similar advantages. Many other differences between normal and neoplastic cells are similar to those between finite and continuous cell lines as immortalization is an important component of the process of transformation.

3.3 Subculture

Freshly isolated cultures are known as primary cultures until they are subcultured. They are usually heterogeneous and have a low growth fraction but are more representative of the cell types in the tissue from which they were derived and in the expression of tissue-specific properties. Subculture allows the expansion of the culture (it is now known as a cell line), the possibility of cloning, characterization and preservation and greater uniformity, but may cause a loss of specialized cells and differentiated properties unless care is taken to select the correct lineage and preserve or reinduce differentiated properties. The greatest advantage of subculturing a primary culture into a cell line is the provision of large amounts of consistent material suitable for prolonged use.

3.3.1 Finite or continuous cell lines?

After several subcultures a cell line will either die out (finite cell line) or 'transform' to become a continuous cell line. It is not clear in all cases whether the stem line of a continuous culture pre-exists or arises during serial propagation. Because of the time taken for such cell

lines to appear (often several months) and the differences in their properties, it has been assumed that a mutation occurs but the pre-existence of immortalized cells particularly in cultures from neoplasms cannot be excluded.

Complementation analysis has shown that senescence is a dominant trait and immortalization the result of mutations and/or deletions in genes such as p53. The activity of telomerase is higher in immortal cell lines and may be sufficient to endow the cell line with an infinite lifespan. The appearance of a continuous cell line is usually marked by an alteration in cytomorphology (giving cells that are smaller, less adherent, more rounded and with a higher ratio of nucleus to cytoplasm) an increase in growth rate (population doubling time can decrease from 36-48 h to 12-36 h), a reduction in serum dependence, an increase in cloning efficiency, a reduction in anchorage dependence (as measured by an increased ability to proliferate in suspension as a liquid culture or cloned in agar), an increase in heteroploidy (chromosomal variation among cells) and aneuploidy (divergence from the donor, euploid, karyotype), and an increase in tumorigenicity. The resemblance between spontaneous *in vitro* transformation and malignant transformation is obvious but nevertheless the two are not necessarily identical although they have much in common. Normal cells can 'transform' to become continuous cell lines without becoming malignant, and malignant tumours can give rise to cultures which 'transform' and become more (or even less) tumorigenic, but acquire the other properties listed above. Transformation *in vitro* is primarily the acquisition of an infinite lifespan. Simultaneous or subsequent alterations in growth control can be under positive control by oncogenes or negative control by tumour suppressor genes.

The advantages of continuous cell lines are their faster growth rates to higher cell densities and resultant greater yield, their lower serum requirement and general ease of maintenance in commercially available media, and their ability to grow in suspension. Their disadvantages include greater chromosomal instability, divergence from the donor phenotype and loss of tissue-specific markers. Examples of commonly used continuous cell lines are given in Table 1.

A number of techniques, including transfection or infection with viral genes (such as E6 and E7 from human papilloma virus, and SV40T) or viruses (such as Epstein-Barr virus, EBV) have been used to immortalize a wide range of cell types. The retention of lineage-sspecific properties is variable.

Table.1.Commonly used Cell Lines

Designation	Species	Tissue	Cell type	Properties
Normal				
MRC-5	Human	Embryo lung	Fibroblastic	Diploid, contact inhibited
3T3-L1	Mouse	Whole embryo	Fibroblastic	contact inhibited, can differentiate into adipose cells
BHK-21	Hamster	Kidney	Fibroblastic	contact inhibited, transformable with polyoma virus
MDCK	Dog	Kidney	Epithelial	Forms 'domes'
Transformed, solid tissue				
293	Human	Embryo kidney	Epithelial	Very sensitive to human adenoviruses
Hep-G2	Human	Hepatoma	Epithelial	Retains some drug metabolizing enzymes
HeLa-S3	Human	Cervical	Epithelial	Rapid growth; high plating efficiency

		carcinoma		
Cos-7	Monkey	Kidney	Epithelial	Good host for transfection
Caco-2	Human	Colo-rectal carcinoma	Epithelial	Forms tight monolayer with polarized transport
Leukemic				
HL-60	Human	Myelocytic	Suspension	Differntiates in esponse to retenoids,NaBt, PMA
Jurkat E6-1	Human	T-Cell	Suspension	Produces IL-2,stimulated by phorbol esters
K562	Human	Erythrocytic	Suspension	Synthesizes haemoglobin when induced with sodium butyrate

3.3.2 Propagation in suspension

Most cultures are propagated as a monolayer attached to the substrate but some including transformed cells, haematopoietic cells, and cells from ascites can be propagated in suspension. Suspension culture has advantages including simpler propagation (subculture only requires dilution, no trypsinization), no requirement for increasing surface area with increasing bulk, ease of harvesting, and the possibility of achieving a 'steady state' or biostat culture if required.

3.4 Selection of medium

Regrettably, the choice of medium is still often empirical. What was used previously by others for the same cells or what is currently being used in the laboratory for different cells, often dictates the choice of medium and serum. For continuous cell lines it may not matter since the conditions are consistent, but for specialized cell types, primary cultures and growth in the absence of serum, the choice is more critical.

There are two major advantages of using more sophisticated media in the absence of serum: they may be selective for particular types of cell, and the isolation of purified products is easier in the absence of serum. Nevertheless, culture in the presence of serum is still easier and often no more expensive, though less controlled.

Two major determinants regulate the use of serum-free media:

(a) *Cost.* Most people do not have the time, facilities or inclination to make up their own media and serum-free formulations, with their various additives tend to be much more expensive than conventional media.
(b) *Requirements for serum-free media are more cell type specific.* Serum will cover many inadequacies revealed in its absence. Furthermore, because of their selectivity, a different medium may be required for each type of cell line. This problem may be particularly acute when culturing tumour cells where cell line variability may require modifications for cell lines from individual tumours.

In the final analysis the choice is often still empirical: read the literature and determine which medium has been used previously. If several media have been used, as is often the case, test them all, with others added if desired (Table 2).

Table.2 Commonly used media

Medium	Properties
RPMI 1640	No Ca^{2+}, low Mg^{2+}; designed for lymphoblastoid and useful for other
MEM/Hanks'	Classic broad specificity medium; low HCO^{-3} for use in air.
MEM/Earles'	Classic broad specificity medium; high HCO_3 for use in 5% CO_2.
F12	Large number of additional constituents (trace elements, copper, iron, additional vitamins, nucleosides, pyruvate, lipoic acid), but at low concentrations; suitable for cloning.
DMEM/F12	50:50 mixture of DMEM and F12; suitable for primary cultures and serum-free with appropriate supplementation.
L15	Bicarbonate-free; buffers in the absence of CO_2.
MCDB 153	One of a series of serum-free media; suitable for growth of keratinocytes

Measure the growth (population doubling time (PDT) and saturation density), cloning efficiency and expression of specific properties (differentiation, transfection efficiency, cell products, etc.). The choice of medium may not be the same in each case, for example differentiation of lung epithelium will proceed in serum but propagation is better without. If possible, include one or more serum-free media in the panel to be tested, supplemented with growth factors, hormones and trace elements as required. Once a medium has been selected, try to keep this constant for as long as possible. Similarly, if serum is used, select a batch by testing samples from commercial suppliers and reserve enough to last six months to one year, before replacing it with another pre-tested batch. Testing procedures are as described above for media selection.

3.5 Gas phase

The composition of the gas phase is determined by:
- The type of medium (principally its sodium bicarbonate concentration).
- Whether the culture vessel is open (Petri dishes, multiwell plates) or sealed (flasks, bottles).
- The amount of buffering required.

Several variables are in play, but one major rule predominates and three basic conditions can be described. The rule is that the bicarbonate concentration and carbon dioxide tension must be in equilibrium. The three conditions are summarized in Table 3. It should be remembered that carbon dioxide/bicarbonate is essential to most cells, so a flask or dish cannot be vented without providing carbon dioxide in the atmosphere.

Prepare medium to about pH 7.1-7.2 at room temperature, incubate a sample with the correct carbon dioxide tension for at least 0.5 h in a shallow dish, and check that the pH stabilizes at pH 7.4. Adjust with sterile 1 M HCl or 1 M NaOH if necessary. Oxygen tension is usually maintained at atmospheric pressure, but variations have been described such as elevated for organ culture and reduced for cloning melanoma and some haematopoietic cells.

Table 3. Relationship between CO_2 and bicarbonate

Open or Closed Vessel	Buffer Caapacity	HCO$_3^-$	Gas Phase	Hepes
Closed Vessel	Low	4 mM	Air	-
Open or Closed Vessel	Moderate	26mM	5% C02	-
Open or Closed Vessel	High	8 mM	2% C02	20 mM

3.6 Culture system

Tissue culture was regarded as the culture of whole fragments of explanted tissue with the assumption that histological integrity was at least partially maintained. Now 'tissue culture' has become a generic term and includes organ culture, where a small fragment of tissue or whole embryonic organ is explanted to retain tissue architecture and cell culture, where the tissue is dispersed mechanically or enzymatically, or the cells migrate from an explant and the cells are propagated as a suspension or attached monolayer. Cell cultures are usually devoid of structural organization, have lost their histotypic architecture and often the biochemical properties associated with it and generally do not achieve a steady state unless special conditions are used. They can, however, be propagated, expanded and divided into identical replicates. They can be characterized and a denned cell population preserved by freezing and they can be purified by growth in selective media, physical cell separation, or cloning to give a characterized cell strain with considerable uniformity.

Organ culture will preserve cell interaction and retain histological and biochemical differentiation for longer than cell culture. After the initial trauma of explantation and some central necrosis, organ cultures can remain in a steady state for a period of several days to years. However, they cannot be propagated easily, show greater experimental variation between replicates and tend to be more difficult to use for quantitative determinations. Purified cell lines can be maintained at high cell density to create histotypic cultures and different cell populations can be combined in organotypic culture, simulating some of the properties of organ culture.

3.6.1 Substrate

The nature of the substrate is determined largely by the type of cell and the use to which it will be put. Polystyrene which has been treated to make it wettable and give it a net negative charge is now used almost universally. In special cases (culture of neurons, muscle cells, capillary endothelial cells, and some epithelial cultures) the plastic is pre-coated with fibronectin, collagen, gelatin, or poly-L-lysine (which gives a net positive charge). Glass may also be used, but must be washed carefully with a non-toxic detergent.

3.6.2 Scale

Culture vessels vary in size from microtitration (30 mm^2, 100-200 µl$)$ up through a range of dishes and flasks to 180 cm^2, and roller bottles and multisurface propagators for large scale culture. The major determinants are the number of cells required (5 X 10^4 to 10^5/cm^2 maximum for most untransformed cells, 105 to 10^6/cm^2 for transformed), the number of replicates (96 or 144 in a microtitration plate), and the times of sampling: a 24-well plate is good for a large number of replicates for simultaneous sampling, but individual dishes, tubes, or bottles are preferable where sampling is carried out at different times. Petri dishes are

cheaper than flasks and good for subsequent processing, e.g., staining or extractions. Flasks can be sealed; do not need a humid carbon dioxide incubator, and give better protection against contamination. Volume is the main determinant for suspension cultures. Sparging (bubbling air through the culture) and agitation will become necessary as the depth Increases.

Where a product is required rather than cells, there are advantages in perfusion systems which can be used to culture cells on membranes or hollow fibres. These supply nutrients across the membrane, and the product is collected either from the cell supernatant or the medium, depending on the molecular weight, which will determine the partitioning of the product on either side of the membrane. Perfusion is also useful for time lapse studies, where cells are monitored on a microscope stage by a video camera, and for pharmacokinetic modelling where the duration and concentration of a test compound can be regulated precisely.

4 Procedures
4.1 Substrate

Most laboratories now utilize disposable plastics as substrates for tissue culture. They are optically clear, prepared for tissue culture use by modification of the plastic to make it wettable and suitable for cell attachment, and come sterilized for use. On the whole they are convenient and provide a reproducible source of vessels for both routine and experimental work.

Some more fastidious cell types, such as bronchial epithelium, vascular endothelium, skeletal muscle, and neurons require the substrate to be coated with extracellular matrix materials such as fibronectin, collagen or laminin. Most matrix products are available individually (Becton Dickinson, Life Technologies, Sigma, Biofluids) or combined in Matrigel (Becton Dickinson), extracellular matrix produced by the Engelberth Holm Swarm sarcoma cell line. Alternatively, the substrate may be coated with extracellular matrix by growing cells on the plastic and then washing them off with 1% Triton X in ultrapure water. Matrix coating can be carried out in three ways:

a. By wetting the surface of the plastic with the matrix component(s), incubating for a short period (usually ~ 30 min), then removing the surplus and using the plastic with adsorbed matrix within seven to ten days (stored at 4°C if not used immediately).
b. By wetting the plastic and removing the surplus matrix material and allowing the residue to dry.
c. By adding collagen or Matrigel and allowing it to gel. Wet or dry coating is used mainly for propagation, while gel coating is used to promote differentiation of cells growing on or in the gel.

4.2 Medium

Most of the commonly used media (Table 2) are available commercially, presterilized. For special formulations or additions it may be necessary to prepare and sterilize some of the constituents. In general, stable solutions (water, salts, and media supplements such as tryptose or peptone) may be autoclaved at 12°C (100 kPa or 1 atmosphere above ambient) for 20 min, while labile solutions (media, trypsin and serum) must be filtered through a 0.2 μm porosity membrane filter (Millipore, Sartorius, Pall Gelman). Sterility testing should be performed on samples of each filtrate. Where an automatic autoclave is used care must be taken to ensure that the timing of the run is determined by the temperature of the centre of the load and not

just by drain or chamber temperature or pressure which will rise much faster than the load. The recorder probe should be placed in a package or bottle of fluid similar to the load and centrally located.

4.3 Cell culture
4.3.1 Primary cultures

The first step in preparing a primary culture is sterile dissection followed by mechanical or enzymatic disaggregation. The tissue may simply be chopped to around 1 mm^3 and the pieces attached to a dish by their own adhesiveness, by scratching the dish, or by using clotted plasma. In these cases cells will grow out from the fragment and may be used directly or subcultured. The fragment of tissue, or explant as it is called, may be transferred to a fresh dish or the outgrowth trypsinized to leave the explant and a new outgrowth generated.

When the cells from the outgrowth are trypsinized and reseeded into a fresh vessel they become a secondary culture, and the culture is now technically a cell line. Primary cultures can also be generated by disaggregating tissue in enzymes such as trypsin (0.25% crude or 0.01-0.05% pure) or collagenase (200-2000 U/ml, crude) and the cell suspension allowed to settle on to, adhere and spread out on the substrate. This type of culture gives a higher yield of cells though it can be more selective, as only certain cells will survive dissociation. In practice, many successful primary cultures are generated using enzymes such as collagenase to reduce the tissue, particularly epithelium, to small clusters of cells which are then allowed to attach and grow out. When primary cultures are initiated, all details of procedures should be carefully documented to form part of the provenance of any cell line that may arise and be found to be important. A sample of tissue, or DNA extracted from it, should be archived to be available for DNA fingerprinting or profiling for authentication of any cell lines that arise.

4.3.2 Subculture

A monolayer culture may be transferred to a second vessel and diluted by dissociating the cells of the monolayer in trypsin (suspension cultures need only be diluted). This is best done by rinsing the monolayer with PBS lacking Ca^{2+} and Mg^{2+} (PBSA) or PBSA containing 1 mM EDTA, and removing the rinse, adding cold trypsin (0.25% crude or 0.01-0.05% pure) for 30 sec, removing the trypsin, and incubating in the residue for 5-15 min, depending on the cell line. Cells are then resuspended in medium, counted and reseeded.

4.3.3 Growth curve

When cells are seeded into a flask they enter a lag period of 2-24 h, followed by a period of exponential growth (the 'log phase'), and finally enter a period of reduced or zero growth after they become confluent ('plateau phase') (Figure 1). These phases are characteristic for each cell line and give rise to measurements which should be reproducible with each serial passage: the length of the lag period, the population doubling time (PDT) in mid-log phase, and the saturation density at plateau, given that the environmental conditions are kept constant. They should be determined when first handling a new cell line and at intervals of every few months thereafter. It is an important element of quality control to be able to demonstrate that the same seeding concentration will yield a reproducible number of cells at subculture, carried out after a consistent time interval, without necessarily performing a growth curve each time. The determination of the growth cycle is important in designing routine subculture and experimental protocols. Cell behaviour and biochemistry changes

significantly at each phase and it is therefore essential to control the stage of the growth cycle when drugs or reagents are added or cells harvested. The shape of the growth curve can also give information on the reproductive potential of the culture where differences in growth rate (PDT) (Figure 1a), adaptation or survival (Figure 1b), and density limitation of growth (level of saturation density in plateau) (Figure 1c), can be deduced from the shape of the curve. However it is generally recognized that the analysis of clonal growth is easier and less prone to ambiguity and misinterpretation (Figure 1d).

4.3.4 Feeding

Some rapidly growing cultures, such as transformed cell lines like HeLa, will require a medium change after three to four days in a seven day subculture cycle. This is usually indicated by an increase in acidity where the pH falls below pH 7.0. Medium can also deteriorate without a major pH change, as some constituents, like glutamine, are unstable, and others may be utilized without a major pH shift. It is, therefore, recommended that the medium is changed at least once per week.

4.3.5 Contamination

The problem of microbial contamination has been greatly reduced by the use of laminar airflow cabinets. The risk of contamination can also be reduced by use of antibiotics, but this should be reserved for high-risk procedures, such as primary culture, and cultures should be maintained in the absence of antibiotics so that chronic, cryptic contaminations are not harboured.

Check frequently for contamination by looking for a rapid change in pH (usually a fall, but some fungi can increase the pH), cloudiness in the medium, extracellular granularity under the microscope, or any unidentified material floating in the medium. If a contamination is detected, discard the flask unopened and autoclave. If in doubt, remove a sample and examine by phase microscopy, Gram's stain, or standard microbiological techniques.

Mycoplasma

Cultures can become contaminated by *Mycoplasma* from media, sera, trypsin, imported cell lines, or the operator. *Mycoplasma* is not visible to the naked eye, and, while they can affect cell growth, their presence is often not obvious. It is important to test for *Mycoplasma* at regular intervals (every one to three months) as they can seriously affect almost every aspect of cell metabolism, antigenicity and growth characteristics. Several tests have been proposed, but the fluorescent DNA staining technique is the most widely used, although it is less sensitive than the PCR-based and culture methods.

4.3.6 Cross-contamination

Its severity is often underrated, and consequently it still occurs with a higher frequency than many people admit. A significant number of cell lines, including Hep-2, KB, and Chang liver, are still in regular use without acknowledgement of their contamination with HeLa. Many other cell lines are cross-contaminated with cells other than HeLa. Many more cross-contaminations are yet to be detected.

To avoid cross-contamination:
• do not share bottles of media or reagents among cell lines
• do not return a pipette, which has been in or near a flask or bottle containing cells, back to the medium bottle; use a fresh pipette
• do not share medium among operatives
• handle one cell line at a time
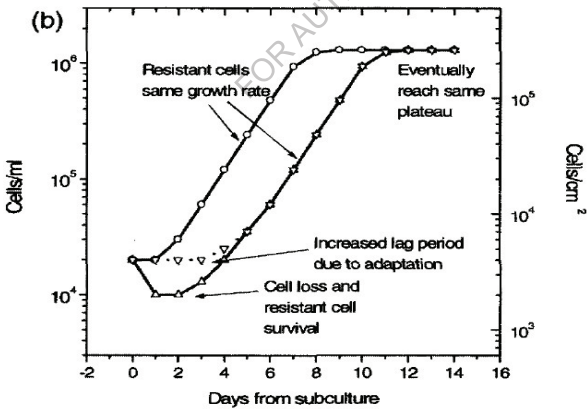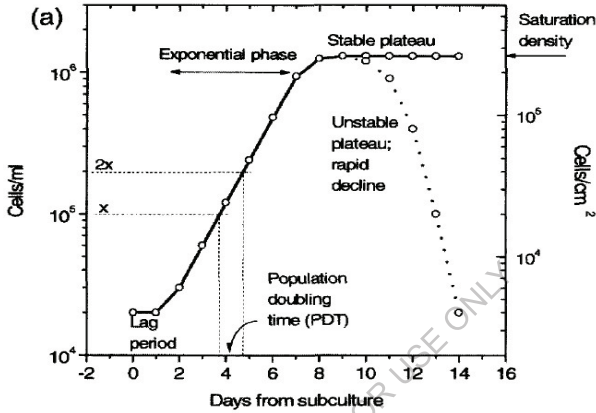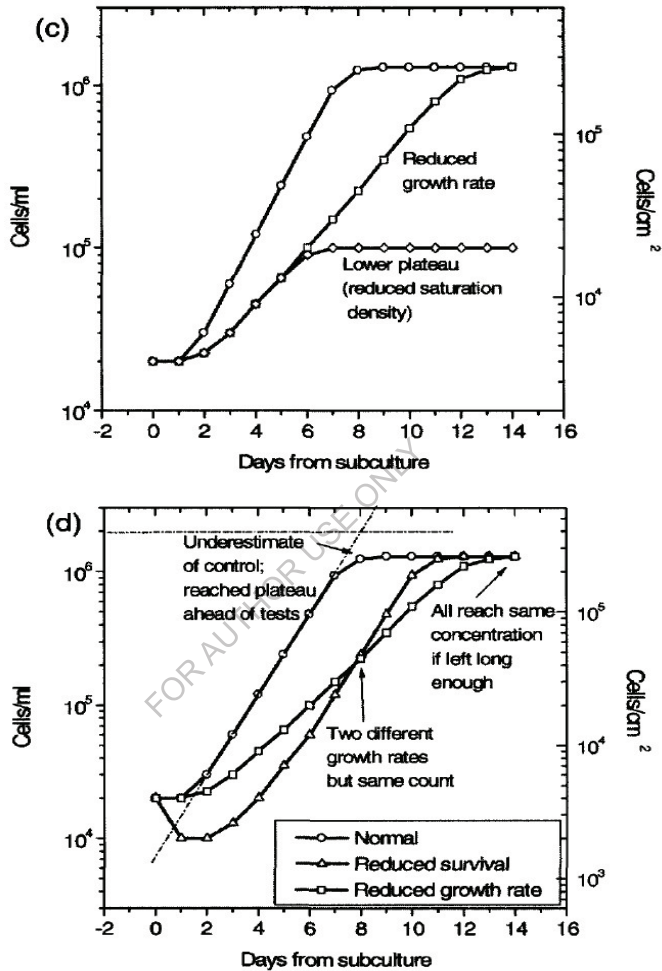
Figure 1. Analysis of growth curves. Examples of growth curves of cells following subculture are shown with cell concentration (cells/ml) on the left axis and cell density (cells/cm^2) on the right axis. Both cell concentration and cell density are important for attached cells and unstirred suspensions, but only concentration is relevant to stirred suspensions. (a) Typical growth curve for a culture seeded at 2 x 10^4 cells/ml (5 x 10^3 cells/cm^2) on day 0, showing a lag period of 24 h, followed by exponential growth ('log' phase) from day 1 to day 7, and entering a plateau phase on, or shortly after, day 8. The solid line represents a stable plateau, seen in most normal cells. The dotted line represents the rapid

deterioration seen with many transformed cell lines, particularly suspension cultures (such as myeloma cultures), where the cells die soon after they have reached the plateau phase. (b) Cells plated as in (a), but in the right-hand curve there is evidence of reduced growth, as the curve displaced to the right. The dip at the beginning (solid line, upward pointing triangles) implies that many cells have not survived but that a resistant fraction has survived and has grown through with the same growth rate as the control. The dotted line (downward pointing triangles) would indicate that there was no cell loss, but that there was a prolonged period of adaptation, followed by recovery at the same growth rate. (c) Cells plated as in (a), showing a reduced growth rate resulting from a longer population doubling time (PDT), with cells reaching same saturation density as control (squares), or plateauing at a lower saturation density (diamonds). Cells could also plateau at a lower saturation density while retaining the same PDT (not shown). (d) Cells plated as in (a), showing potential misinterpretations of data indicating reduced growth rates. The curve with upward facing triangles shows a reduced survival but the same growth rate. The curve denoted by squares shows a reduced PDT. Both curves converge at 8 days in this example, but could converge at any time with appropriate PDT and survival, indicating that single time points are unsuitable for correct analysis. In this example, the 8 day point could overestimate growth as the control has started to plateau. Although this occurs at 8 days in this example, it could occur earlier with a more rapidly growing cell line. Furthermore, all curves converge at the same saturation density if left long enough. Meaningful comparative observations can only be made while all cells are in exponential growth.

4.3.7 Instability and preservation

Early passage cell lines are unstable as they go through a period of adaptation to culture. However, between about the 5th and 35th generation (for human diploid fibroblasts; other cell types may be different) the culture is fairly consistent. As the culture will start to senesce as it gets older, finite cell lines should be used after the period of adaptation but before senescence. As continuous cell lines can be genetically unstable they should only be used continuously for approximately three months, before stock replacement. Some cell lines, such as 3T3 and other mouse lines which are immortal but not transformed, can transform spontaneously, and should not be propagated for prolonged periods. Validated and authenticated frozen stocks of all cell lines should be maintained to protect against cell line instability, and to give insurance against contamination, incubator failure, or other accidental loss.

Animal cell culture is a precise discipline. Beware of those who say it is not, or it is 'magic', or due to 'green fingers'; they are not controlling all the variables. Consistency can be achieved, and the following chapters are intended to indicate how best to control conditions within the present limitations of our knowledge.

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Chapter 3
Scaling-up of animal cell cultures

1 Introduction

Small scale culture of cells in flasks of up to 1 litre volume (175 cm^2 surface area) is the best means of establishing new cell lines in culture, for studying cell morphology and for comparing the effects of agents on growth and metabolism. However, there are many applications in which large numbers of cells are required, for example extraction of a cellular constituent (10^9 cells can provide 7 mg DNA); to produce viruses for vaccine production (typically 5 x 10^{10} cells per batch) or other cell products (interferon, plasminogen activator, interleukins, hormones, enzymes, erythropoietin, and antibodies); and to produce inocula for even larger cultures. Animal cell culture is a widely used production process in biotechnology, with systems in operation at scales over 10000 litres. This has been achieved by graduating from multiples of small cultures, an approach which is tedious, labour-intensive and expensive to the use of large 'unit process' systems. Although unit processes are more cost-effective and efficient, achieving the necessary scale-up has required a series of modifications to overcome limiting factors such as oxygen limitation, shear damage, and metabolite toxicity. One of the aims of this chapter is to describe these limitations, indicate at what scale they are likely to occur, and suggest possible solutions. This theme has to be applied to cells that grow in suspension and to those that will only grow when attached to a substrate (anchorage-dependent cells). Another aspect of scale-up that will be discussed is increasing unit cell density 50- to 100-fold by the use of cell immobilization and perfusion techniques. Free suspension culture offers the easiest means of scale-up because a 1 litre vessel is conceptually very similar to a 1000 litre vessel. The changes concern the degree of environmental control and the means of maintaining the correct physiological conditions for cell growth, rather than significantly altering vessel design. Monolayer systems (for anchorage-dependent cells) are more difficult to scale-up in a single vessel, and consequently a wide range of diverse systems have evolved. The aim of increasing the surface area available to the cells and the total culture volume has been successfully achieved, and the most effective of these methods (microcarrier) will be discussed.

2 General methods and culture parameters

Familiarity with certain biological concepts and methods is essential when understanding scale-up of a culture system. In small scale cultures there is leeway for some error. If the culture fails it is a nuisance, but not necessarily a disaster. Large scale culture failure is not only more serious in terms of cost, but also the system demands that conditions are more critically met. This section describes the factors that need to be considered as the culture size gets larger.

2.1 Cell quantification

Measuring total cell numbers (by haemocytometer counts of whole cells or stained nuclei) and total cell mass (by determining protein or dry weight) is easily achieved. It is far more difficult to get a reliable measure of cell viability because the methods used either stress the cells or use a specific and not necessarily typical, parameter of cell physiology. An additional difficulty is that in many culture systems the cells cannot be sampled (most

anchoragedependent cultures), or visually examined, and an indirect measurement has to be made.

2.1.1 Cell viability

The dye exclusion test is based on the concept that viable cells do not take up certain dyes, whereas dead cells are permeable to these dyes. Trypan blue (0.4%) is the most commonly used dye, but has the disadvantage of staining soluble protein. In the presence of serum, therefore, erythrocin B (0.4%) is often preferred. Cells are counted in the standard manner using a haemocytometer. Some caution should be used when interpreting results as the uptake of the dye is pH and concentration-dependent and there are situations in which misleading results can be obtained. Two relevant examples are membrane leakiness caused by recent trypsinization and freezing and thawing in the presence of dimethyl sulfoxide. A colorimetric method using the MTT assay can be used both to measure viability after release of cytoplasmic contents into the medium from artificially lysed cells, and for microscopic visualization within the attached cell.

2.1.2 Indirect measurements

Indirect measurements of viability are based on metabolic activity. The most commonly used parameter is glucose utilization, but oxygen utilization, lactic or pyruvic acid production, or carbon dioxide production can also be used, as can the expression of a product, such as an enzyme. When cells are growing logarithmically, there is a very close correlation between nutrient utilization and cell numbers. However, during other growth phases, utilization rates, caused by maintenance rather than growth, can give misleading results. The measurements obtained can be expressed as a growth yield (Y) or specific utilization/respiration rate (Q):

$$\text{Growth yield (Y)} = \frac{\text{change in biomass concentration (dx)}}{\text{change in substrate concentration (ds)}} \qquad 1$$

$$\text{Specific utilization/respiration rate (}Q_A\text{)} =$$
$$\frac{\text{change in substrate concentration (ds)}}{\text{time (dt)} \times \text{cell mass/numbers (dx)}} \qquad 2$$

Typical values of growth yields for glucose (10^6 cells/g) are 385 (MRC-5), 620 (Vero), and 500 (BHK).

A method which is not so influenced by growth rate fluctuations is the lactate dehydrogenase (LDH) assay. LDH is measured in cell-free medium at 30°C by following the oxidation of NADH by the change in absorbance at 340 nm. The reaction is initiated by the addition of pyruvate. One unit of activity is defined as 1 umol/min NADH consumed. LDH is released by dead/dying cells and is therefore a quantitative measurement of loss of cell viability. To measure viable cells a reverse assay can be performed by controlled lysis of the cells and measuring the increase in LDH.

2.2 Equipment and reagents
2.2.1 Culture vessel and growth surfaces

The standard non-disposable material for growth of animal cells is glass, although this is replaced by stainless steel in larger cultures. It is preferable to use borosilicate glass (e.g.,

Pyrex) because it is less alkaline than soda glass and withstands handling and autoclaving better. Cells usually attach readily to glass but, if necessary, attachment may be augmented by various surface treatments. In suspension culture, cell attachment has to be discouraged, and this is achieved by treatment of the culture vessel with a proprietary silicone preparation (siliconization). Examples are Dow Corning 1107 (which has to be baked on) or dimethyldichlorosilane (Repelcote, Hopkins and Williams) which requires thorough washing of the vessel in distilled water to remove the trichloroethane solvent.

Complex systems use a combination of stainless steel and silicone tubing to connect various components of the system. Silicone tubing is very permeable to gases, and loss of dissolved carbon dioxide can be a problem. It is also liable to rapid wear when used in a peristaltic pump. Thick-walled tubing with additional strengthening (sleeve) should be used. Custom-made connectors should be used to ensure good aseptic connection during process operation. Safe removal of samples of the culture at frequent intervals is essential. An entry with a vaccine stopper through which a hypodermic syringe can be inserted provides a simple solution, but is only suitable for small cultures. Repeated piercing of the vaccine stopper can lead to a loss of culture integrity. The use of specialized sampling devices, also available from fermenter supply companies, is recommended. These automatically enable the line to be cleared of static medium containing dead cells, thus avoiding the necessity of taking small initial samples which are then discarded, and increasing the chances of retaining sterility. Air filters are required for the entry and exit of gases. Even if continuous gassing is not used, one filter entry is usually needed to equilibrate pressure and for forced input or withdrawal of medium. The filters should have a 0.22 um rating and be non-wettable.

2.2.2 Non-nutritional medium supplements

Sodium carboxymethylcellulose (15-20 centrepoise, units of viscosity) is often added to media (at 0.1%) to help minimize mechanical damage to cells caused by shear forces generated by the stirrer impeller, forced aeration, or perfusion. This compound is more soluble than methylcellulose, and has a higher solubility at 4°C than at 37°C.

Pluronic F-68 (trade name for polyglycol) (BASF, Wyandot) is often added to media (at 0.1%) to reduce the amount of foaming that occurs in stirred and/or aerated cultures, especially when serum is present. It is also helpful in reducing cell attachment to glass by suppressing the action of serum in the attachment process. However, its most beneficial action is to protect cells from shear stress and bubble damage in stirred and sparged cultures, and it is especially effective in low serum or serum-free media.

2.3 Practical considerations

- *Temperature of medium.* Always pre-warm the medium to the operating temperature (usually 37°C) and stabilize the pH before adding cells. Shifts in pH during the initial stages of a culture create many problems, including a long lag phase and reduced yield.
- *Growth phase of cells.* Avoid using stationary phase cells as inoculums because this will mean a long lag phase or no growth at all. Ideally, cells in the late logarithmic phase should be used.
- *Inoculation density.* Always inoculate at a high enough cell density. There is no set rule as to the minimum inoculum level below which cells will not grow, as this varies

between cell lines and depends on the complexity of the medium being used. As a guide, it may be between 5×10^4 and 2×10^5 cells/ml or 5×10^3 and 2×10^4 cells/cm^2.

- **Stirring rate**. Find empirically the optimum stirring rate for a given culture vessel and cell line. This could vary between 100-500 r.p.m. for suspension cells, but is usually in the range 200-350 r.p.m., and between 20-100 r.p.m. for microcarrier cultures.
- **Medium and surface area**. The productivity of the system depends upon the quality and quantity of the medium and, for anchorage-dependent cells, the surface area for cell growth. A unit volume of medium is only capable of giving a finite yield of cells. Factors which affect the yield are: pH, oxygen limitation, accumulation of toxic products (e.g., NH$_4$), nutrient limitation (e.g., glutamine), spatial restrictions, and mechanical/shear stress. As soon as one of these factors comes into effect, the culture is finished and the remaining resources of the system are wasted. The aim is, therefore, to delay the onset of any one factor until the accumulated effect causes cessation of growth, at which point the system has been maximally utilized. Simple ways of achieving this are: a better buffering system (e.g., Hepes instead of bicarbonate), continuous gassing, generous headspace volume, enriched rather than basal media, with nutrient-sparing supplements such as non-essential amino acids or lactalbumin hydrolysate, perfusion loops through ultrafiltration membranes or dialysis tubing for detoxification and oxygenation, and attention to culture and process design.

2.4 Growth kinetics

The standard format of a culture cycle beginning with a lag phase, proceeding through the logarithmic phase to a stationary phase and finally to the decline and death of cells, is well documented. Although cell growth usually implies increase in cell numbers, increase in cell mass can occur without any replication. The difference in mean cell mass between cell populations is considerable, as would be expected, but so is the variation within the same population. Growth (increase in cell numbers or mass) can be defined in the following terms.

(a) Specific growth rate, μ (i.e., the rate of growth per unit amount (weight/numbers) of biomass):

$$\mu = (1/x)\,(dx/dt)h^{-1} \qquad\qquad 3$$

where dx is the increase in cell mass, dt is the time interval, and x is the cell mass. If the growth rate is constant (e.g., during logarithmic growth), then

$$\ln x = \ln x_0 + \mu t \qquad\qquad 4$$

where X_0 is the biomass at time t_0

(b) Doubling time, td (i.e., the time for a population to double in number/mass):

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad\qquad 5$$

(c) Degree of multiplication, n or number of doublings (i.e., the number of times the inoculum has replicated):

$$n = 3.32 \log(x/x_0) \qquad\qquad 6$$

2.5 Medium and nutrients

A given concentration of nutrients can only support a certain number of cells. Alternative nutrients can often be found by a cell when one becomes exhausted, but this is bad practice because the growth rate is always reduced (e.g., while alternative enzymes are being induced). If a minimal medium, such as Eagle's basal medium (BME) or minimum essential medium (MEM) is used with serum as the only supplement, then this problem is going to be met sooner than in cultures using complete media (e.g., 199), or in media supplemented with lactalbumin hydrolysate, peptone, or BSA (which provides many of the fatty acids). Nutrients likely to be exhausted first are glutamine, partly because it spontaneously cyclizes to pyrrolidone carboxylic acid and is enzymically converted (by serum and cellular enzymes) to glutamic acid, leucine, and isoleucine. Human diploid cells are almost unique in utilizing cystine heavily. A point to remember is that nutrients become growth-limiting before they become exhausted. As the concentration of amino acids falls, the cell finds it increasingly difficult to maintain sufficient intracellular pool levels. This is exaggerated in monolayer cultures because as cells become more tightly packed together, the surface area available for nutrient uptake becomes smaller. Glucose is often another limiting factor as it is destructively utilized by cells and, rather than adding high concentrations at the beginning, it is more beneficial to supplement after two to three days. To maintain a culture, some additional feeding often has to be carried out either by complete, or partial, media changes or by perfusion. Many cell types are either totally dependent upon certain additives or can only perform optimally when they are present. For many purposes it is highly desirable, or even essential, to reduce the serum level to 1% or below. To achieve this without a significant reduction in cell yield, various growth factors and hormones are added to the basal medium. The most common additives are insulin (5 mg/litre), transferrin (5-35 mg/litre), ethanolamine (20 μM), and selenium (5 μg/litre). Cell aggregation is often a problem in suspension cultures. Media lacking calcium and magnesium ions have been designed specifically for suspension cells because of the role of these ions in attachment. This problem has also been overcome by including very low levels of trypsin in the medium (2 μg/ml).

2.6 pH

Ideally pH should be near 7.4 at the initiation of a culture and not fall below a value of 7.0 during the culture, although many hybridoma lines appear to prefer a pH of 7.0 or lower. A pH below 6.8 is usually inhibitory to cell growth. Factors affecting the pH stability of the medium are buffer capacity and type, headspace, and glucose concentration. The normal buffer system in tissue culture media is the carbon dioxide bicarbonate system analogous to that in blood. This is a weak buffer system, in that it has a pKa well below the physiological optimum. It also requires the addition of carbon dioxide to the headspace above the medium to prevent the loss of carbon dioxide and an increase in hydroxyl ions. The buffering capacity of the medium is increased by the phosphates present in the balanced salt solution (BSS). Medium intended to equilibrate with 5% carbon dioxide usually contain Earle's BSS (25 mM NaHCO3) but an alternative is Hanks' BSS (4 mM NaHCO3) for equilibration with air. Improved buffering and pH stability in media is possible by using a zwitterionic buffer such as Hepes (10-20 mM), either in addition to, or instead of, bicarbonate (include bicarbonate at

0.5 mM). Alternative buffer systems are provided by using specialist media such as Leibovitz's L-15. This medium utilizes the buffering capacity of free amino acids, substitutes galactose and pyruvate for glucose, and omits sodium bicarbonate. It is suitable for open cultures.

The headspace volume in a closed culture is important because, in the initial stages of the culture, 5% carbon dioxide is needed to maintain a stable pH in the medium but, as the cells grow and generate carbon dioxide, it builds up in the headspace and this prevents it diffusing out of the medium. The result is an increase in weakly dissociated bicarbonate producing an excess of hydrogen ions in the medium and a fall in pH. Thus, a large headspace is required in closed cultures, typically tenfold greater than the medium volume (this volume is also needed to supply adequate oxygen). This generous headspace is not possible as cultures are scaled-up, and an open system with a continuous flow of air, supplied through one filter and extracted through another, is required. The metabolism of glucose by cells results in the accumulation of pyruvic and lactic acids. Glucose is metabolized at a far greater rate than it is needed. Thus, glucose should ideally never be included in media at concentrations above 2 g/litre, and it is better to supplement during the culture than to increase the initial concentration. An alternative is to substitute glucose by galactose or fructose as this significantly reduces the formation of lactic acid, but it usually results in a slower growth rate. These precautions delay the onset of a non-physiological pH and are sufficient for small cultures. As scale-up increases, headspace volume and culture surface area in relation to the medium volume decrease. Also, many systems are developed to increase the surface area for cell attachment and cell density per unit volume. Thus pH problems occur far earlier in the culture cycle because carbon dioxide cannot escape as readily, and more cells means higher production of lactic acid and carbon dioxide. The answer is to carry out frequent medium changes or use perfusion, or have a pH control system. The basis of a pH control system is an autoclavable pH probe (available from Pye Ingold, Russell). This feeds a signal to the pH controller which is converted to give a digital or analogue display of the pH. This is a pH monitor system. Control of pH requires the defining of high and low pH values beyond which the pH should not go. These two set points on the pH scale turn on a relay to activate a pump, or solenoid valve, which allows additions of acid or alkali to be made to the culture to bring it back to within the allowable units. It is rare to have the pH rise above the set point once the medium has equilibrated with the headspace, so the addition of acid can be disregarded. If an alkali is to be added, then sodium bicarbonate (5.5%) is recommended. Sodium hydroxide (0.2 M) can be used only with a fast stirring rate, which dilutes the alkali before localized concentrations can damage the cells, or if a perfusion loop is installed. Normally, liquid delivery pumps are supplied as part of the pH controller. Gas supply is controlled by a solenoid valve and 95% air directly. Above the set point carbon dioxide will be mixed with the air, but below this point only air will be delivered to the culture. This in itself is a controlling factor in that it helps remove carbon dioxide as well as meeting the oxygen requirements of the cultures. pH regulation is very readily adapted to computer control systems.

2.7 Oxygen

The scale-up of animal cell cultures is very dependent upon the ability to supply sufficient oxygen without causing cell damage. Oxygen is only sparingly soluble in culture media (7.6 µg/ml) and a survey of reported oxygen utilization rates by cells reveals a mean value of 6 µg /10^6 cells/h. A typical culture of 2 x 10^6 cells/ml would, therefore, deplete the oxygen content of the medium (7.6 µg/ml) in under 1 h. It is necessary to supply oxygen to

the medium throughout the life of the culture and the ability to do this adequately depends upon the oxygen transfer rate (OTR) of the system:

$$OTR = Kla\ (C^* - C) \qquad\qquad 7$$

where OTR is the amount of oxygen transferred per unit volume in unit time, Kl is the oxygen transfer coefficient, and a is the area of the interface across which oxygen transfer occurs (as this can only be measured in stationary and surfaceaerated cultures, the value JCla is used; this is the mass transfer coefficient (vol/h). C* is the concentration of dissolved oxygen when medium is saturated, and C is the actual concentration of oxygen at any given time. The Kla (OTR/C when C = 0) is in units of h"1 hours"1 and is thus a measure of the time taken to oxygenate a given culture vessel completely under a particular set of conditions.

A culture can be aerated by one or a combination of the following methods: surface aeration, sparging, membrane diffusion, medium perfusion, increasing the partial pressure of oxygen, and increasing the atmospheric pressure.

2.7.1 Surface aeration: static cultures

In a closed system, such as a sealed flask, the important factors are the amount of oxygen in the system and the availability of this oxygen to the cells growing under 3-6 mm of medium. Normally a headspace/medium volume ratio of 10:1 is used to provide sufficient oxygen. Thus a 1 litre flask (e.g., a Roux bottle) with 900 ml of air and 100 ml of medium will initially contain 0.27 g oxygen (Table 1). This amount will support 10^8 cells for 450 h and is thus clearly adequate. The second factor is whether this oxygen can be made available to the cells. The transfer rate of oxygen from the gas phase into a liquid phase has been calculated at 17 jug/cm^2/h. Again, this is well in excess of that required by cells in a 1 litre flask. However, if the surface is assumed to be saturated with dissolved oxygen and the concentration at the cell sheet is almost zero then, applying Fick's law of diffusion, the rate at which oxygen can diffuse to the cells is about 1.5 µg /cm^2/h. At this rate there is only sufficient oxygen to support about 50×10^6 cells in a 1 litre flask, a cell density which in practice many tissue culturists take as the norm. These calculations show the importance of maintaining a large headspace volume; otherwise, oxygen limitation could become one of the growth-limiting factors in static closed cultures.

Table 1. Oxygen concentrations on the gas and liquid phases of a Roux bottle culture

Oxygen In 900 ml air: 900 X 0.21 X 32/22400 = 0.27 g
Oxygen In 100 ml medium: 100 X 7.6 X 10^{-4} = 0.0076 g
Notes:
0.21 = proportion of oxygen in air;
32 = molecular weight of oxygen;
22400 = gram molecular volume;
x 10^{-4} = solubility of oxygen in water at 37°C when equilibrated with air.

2.7.2 Sparging

This is the bubbling of gas through the culture and is a very efficient means of effecting oxygen transfer (as proven in bacterial fermentation). However, it may be damaging to

animal cells due to the effect of the high surface energy of the bubble on the cell membrane. This damaging effect can be minimized by using large air bubbles (which have lower surface energies than small bubbles), by using a very low gassing rate (e.g., 5 ml/1 min), and by adding Pluronic F-68. A specialized form of sparging, the airlift fermenter has also been used in large unit process monolayer cultures (e.g., multiple plate propagators). When sparging is used, efficiency of oxygenation is increased by using a culture vessel with a large height/diameter ratio. This creates a higher pressure at the base of the reactor, which increases oxygen solubility.

2.7.3 Membrane diffusion

Silicone tubing is very permeable to gases, and if long lengths of thin-walled tubing can be arranged in the culture vessel then sufficient diffusion of oxygen into the culture can be obtained. However, a lot of tubing is required (e.g., 30 m of 2.5 cm tubing for a 1000 litre culture). This method is expensive and inconvenient to use, and has the inherent problem that scale-up of the tubing required is mainly two-dimensional while that of the culture is three-dimensional. However, several commercial systems are available (e.g., Braun).

2.7.4 Medium perfusion

A closed-loop perfusion system continuously (or on demand) takes medium from the culture, passes it through an oxygenation chamber, and returns it to the culture. This method has many advantages if the medium can be conveniently separated from the cells for perfusion through the loop. The medium in the chamber can be vigorously sparged to ensure oxygen saturation and other additions, such as sodium hydroxide for pH control, which would damage the cells if put directly into the culture, can be made. This method is used in glass bead systems and has proved particularly effective in microcarrier systems, where specially modified spin filters can be used.

2.7.5 Environmental supply

The dissolved oxygen concentration can be increased by increasing the headspace pO_2 (from atmospheric 21% to any value, using oxygen and nitrogen mixtures) and by raising the pressure of the culture by 100 kPa (about 1 atm) (which increases the solubility of oxygen and its diffusion rate). These methods should be used only when the culture is well advanced, otherwise oxygen toxicity could occur. Finally, the geometry of the stirrer blade also affects the oxygen transfer rate.

2.7.6 Scale-up

Oxygen limitation is usually the first factor to be overcome in culture scale-up. This becomes a problem in conventional stirred cultures at volumes above 10 litres. However, with the current use of high density cultures maintained by perfusion, oxygen limitation can occur in a 2 litre culture. The relative effectiveness of some of the alternative oxygenation systems in large scale bioreactors is shown in Table 2, and the range of oxygenation procedures in various types of culture vessels has been reviewed.

Table 2. Methods of oxygenating a 40 litre bioreactor (30 litre working volume with a 1.5:1 aspect ratio)

Oxygenating method	Oxygen delivery(mg/litre/h)	No. cells x 10^6/ml supported
Air (10 ml/min at 40 r.p.m.)		
Surface aeration	0.5	0.08
Direct sparging	4.6	0.76
Spin filter sparging	3	0.4
Perfusion (1 vol/h)	12.6	2.1
Perfusion (1 vol/h)+ spin filter sparging	15.9	2.65
Oxygen (10 ml/mln at 80 r.p.m.)		
Spin filter sparging	51	8.5
+ perfusion (1 vol/h) (assuming oxygen utilization rate of 2-6 µg /10^6 cells/h).	92	15

2.7.7 Redox potential

The oxidation-reduction potential (ORP) or redox potential, is a measure of the charge of the medium and is affected by the proportion of oxidative and reducing chemicals, the oxygen concentration, and the pH. When fresh medium is prepared and placed in the culture vessel it takes time for the redox potential to equilibrate, a phenomenon known as poising. The optimum level for many cell lines is +75 mV, which corresponds to a dissolved pO$_2$ of 8-10%. Some investigators find it beneficial to control the oxygen supply to the culture by redox, rather than an oxygen electrode. Alternatively, if the redox potential is monitored by a redox electrode and pH meter (with mV display) then an indication of how cell growth is progressing can be obtained. This is because the redox value falls during logarithmic growth and reaches a minimum value approximately 24 h before the onset of the stationary phase (Figure 1). This provides a useful guide to cell growth in cultures where cell sampling is not possible. It is also useful to be able to predict the end of the logarithmic growth phase so that medium changes, addition of virus, or product promoters can be given at the optimum time.
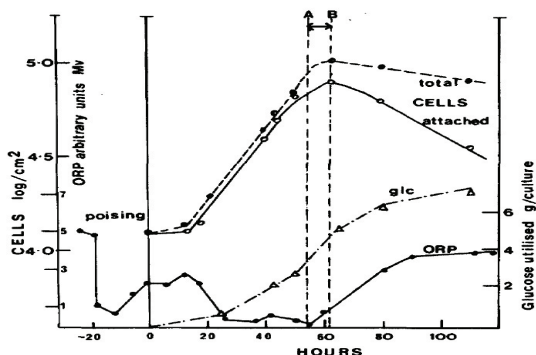
Figure 1. Changes in oxygen-reduction potential (ORP) correlated to cell growth and glucose (glc) utilization. The minimum ORP value (A) is reached 24 h before the end of

logarithmic growth indicated by the maximum cell number (B). The upper curve shows the total number of cells, while the lower curve shows the number of attached cells.

2.8 Types of culture process
2.8.1 Batch and continuous culture

In standard culture, known as batch culture, cells are inoculated into a fixed volume of medium and, as they grow, nutrients are consumed and metabolites accumulate. The environment is therefore continually changing, and this in turn enforces changes to cell metabolism, often referred to as physiological differentiation. Eventually cell multiplication ceases because of exhaustion of nutrient(s), accumulation of toxic waste products, or density-dependent limitation of growth in monolayer cultures. There are means of prolonging the life of a batch culture, and thus increasing the yield, by various substrates feed methods.

a. Gradual addition of fresh medium, so increasing the volume of the culture (fed batch).
b. Intermittently, by replacing a constant fraction of the culture with an equal volume of fresh medium (semi-continuous batch). All batch culture systems retain the accumulating waste products, to some degree, and have a fluctuating environment. All are suitable for both monolayer and suspension cells.
c. Perfusion, by the continuous addition of medium to the culture and the withdrawal of an equal volume of used (cell-free) medium. Perfusion can be open, with complete removal of medium from the system, or closed, with recirculation of the medium, usually via a secondary vessel, back into the culture vessel. The secondary vessel is used to "regenerate" the medium by gassing and pH correction.
d. Continuous-flow culture, which gives true homeostatic conditions with no fluctuations of nutrients, metabolites, or cell numbers. It depends upon medium entering the culture with a corresponding withdrawal of medium plus cells. It is thus only suitable for suspension culture cells, or monolayer cells growing on microcarriers.

2.8.2 Comparison of batch, perfusion, and continuous-flow culture

Continuous-flow culture is the only system in which the cellular content is homogeneous, and can be kept homogeneous for long periods of time (months). This can be vital for physiological studies, but may not be the most economical method for product generation. Production economics are calculated in terms of staff time, medium, equipment, and downstream processing costs. Also taken into account are the complexity and sophistication of the equipment and process, as this affects the calibre of the staff required and the reliability of the production process. Batch culture is more expensive on staff time and culture ingredients, because for every single harvest a sequence of inoculum build-up steps and then growth in the final vessel has to be carried out, and there is also downtime whilst the culture is prepared for its next run. Feeding routines for batch cultures can give repeated but smaller harvests and the longer a culture can be maintained in a productive state then the more economical the whole process becomes (Figure 2).
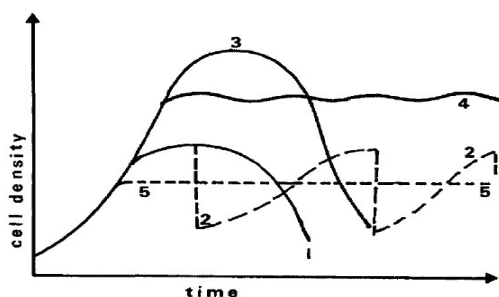
Figure 2. Comparison of culture processes.

The numbers 1-5 are explained in the table below.

Culture type	Cell no.(millions)	Product yield per litre	
		mg/week*	mg/month*
1 Batch	3	6	12
2 Semi-continuous batch	3	15	60
3 Fed batch	30	60	120
4 Continuous perfusion	10	70	280
5 Continuous flow	2	15	60

* Values allow for turn-around time of non-continuous cultures

Continuous-flow culture in the chemostat implies that cell yields are never maximal because a limiting growth factor is used to control the growth rate. If maximum yields are desired in this type of culture then the turbidostat option has to be used. Some applications, such as the production of a cytopathic virus, leave no choice other than batch culture. Maintenance of high yields, and therefore high product concentration, may be necessary to reduce downstream processing costs and these could outweigh medium expenses. For this purpose perfusion has to be used. Although for many processes this is more economical than batch culture, it does add to the complexity of the equipment and process, and increases the risk of a mechanical or electrical failure or microbial contamination prematurely ending the production run. There is no clear-cut answer to which type of culture process should be used-it depends upon the cell and product, the quantity of product, downstream processing problems, and product licensing regulations (batch definition of product, cell stability, and generation number). However, a relative ratio of unit costs for perfusion, continuous-flow, and batch culture in the production of monoclonal antibody is 1:2:3.5.

2.9 Summary of factors limiting scale-up

Apart from scale and different growth characteristics of cells, is the range of solutions which have been used to overcome limitations to scale-up. These will be briefly reviewed so that the underlying philosophy and evolution of the culture systems. As already explained, oxygen is the first limiting factor encountered in scaleup. To overcome this, reactors are

available with membrane or hollow fibre devices to give bubble-free aeration, often by external medium loops. A related factor is mixing, and low-shear options such as airlift reactors and specially designed impellers have been developed. For anchorage-dependent cells the low unit surface area has limited effective scale-up, so a range of devices using plates, spirals, ceramics and, most effective of all, microcarriers, can be used.

The next problem to be overcome is nutrient limitation and toxic metabolite build-up. The most effective solution is to use perfusion by spin filters or hollow fibre loops. To maximize the benefit of perfusion, immobilization of suspension cells was needed to prevent cell wash-out, and this resulted in a collection of novel systems based on hollow fibres, membranes, encapsulation, and specialized matrices. Immobilization has many process advantages, and currently the effort is on scaling-up suitable entrapment matrices in fluidized and fixed beds.

3 Monolayer culture
3.1 Introduction

Tissue culture flasks and tubes giving surface areas of 5-200 cm^2 are familiar to all tissue culturists. The largest stationary flask routinely used in laboratories is the Roux bottle (or disposable plastic equivalent) which gives a surface area for cell attachment of 175-200 cm^2 (depending upon type), needs 100-150 ml medium, and utilizes 750-1000 cm^2 of storage space. This vessel will yield 2 x 10^7 diploid cells and up to 10^8 heteroploid cells. If one has to produce, for example, 10^{10} cells, then over 100 replicate cultures are needed (i.e., manipulations have to be repeated 100 times). In addition the cubic capacity of incubator space needed is over 100 litres. Clearly there comes a time in the scale-up of cell production when one has to use a more efficient culture system. Scale-up of anchorage-dependent cells reduces the number of cultures, is more efficient in the use of staff, and increases significantly the surface area/volume ratio. To do this a very wide and versatile range of tissue culture vessels and systems has been developed. Many of these are shown diagrammatically in Figure 3. The methods with the most potential are those based on modifications to suspension culture systems because they allow a truly homogeneous unit process with enormous scale-up potential to be used. However, these systems should be attempted only if time and resources allow a lengthy development period.
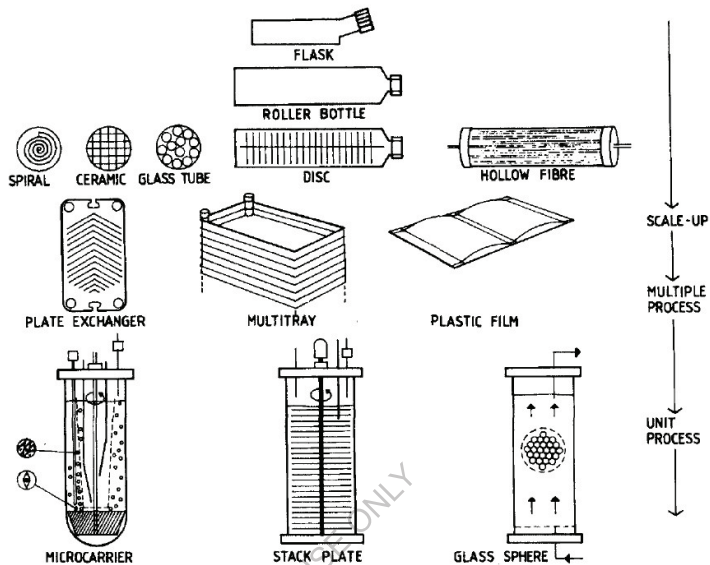
Figure 3. The scaling-up of culture systems for anchorage-dependent cells

Although suspension culture is the preferred method for increasing capacity, monolayer culture has the following advantages:

a. It is very easy to change the medium completely and to wash the cell sheet before adding fresh medium. This is important in many applications when the growth is carried out in one set of conditions and product generation in another. A common requirement of a medium change is the transfer of cells from serum to serum-free conditions. The efficiency of medium changing in monolayer cultures is such that a total removal of the unwanted compound can be achieved.

b. If artificially high cell densities are needed then these can be supported by using perfusion techniques. It is much easier to perfuse monolayer cultures because they are immobilized and a fine filter system (to withhold cells) is not required.

c. Many cells will express a required product more efficiently when attached to a substrate.

d. The same apparatus can be used with different media/cell ratios which, of course, can be easily changed during an experiment.

e. Monolayer cultures are more flexible because they can be used for all cell types. If a variety of cell types are to be used then a monolayer system might be a better investment. It should be noted that the microcarrier system confers some of the advantages of a suspension culture system.

There are four main disadvantages of monolayer compared to suspension systems:

1. They are difficult and expensive to scale-up.
2. They require far more space.

3. Cell growth cannot be monitored as effectively, because of the difficulty of sampling and counting an aliquot of cells.
4. It is more difficult to measure and control parameters such as pH and oxygen, and to achieve homogeneity.

Microcarrier culture eliminates or at least reduces many of these disadvantages.

3.2 Cell attachment

Animal cell surfaces and the traditional glass and plastic culture surfaces are negatively charged, so for cell attachment to occur, crosslinking with glycoproteins and/or divalent cations (Ca^{2+}, Mg^{2+}) is required. The glycoprotein most studied in this respect is fibronectin, a compound of high molecular mass (220 000) synthesized by many cells and present in serum and other physiological fluids. Although cells can presumably attach by electrostatic forces alone, it has been found that the mechanism of attachment is similar, whatever the substrate charge. The important factor is the net negative charge, and surfaces such as glass and metal which have high surface energies are very suitable for cell attachment. Organic surfaces need to be wettable and negative, and this can be achieved by chemical treatment (e.g., oxidizing agents, strong acids) or physical treatment (e.g., high voltage discharge, UV light, high energy electron bombardment). One or more of these methods are used by manufacturers of tissue culture grade plastics. The result is to increase the net negative charge of the surface (for example by forming negative carboxyl groups) for electrostatic attachment.

Surfaces may also be coated to make them suitable for cell attachment. A tissue culture grade of collagen can be purchased which saves a tedious preparation procedure using rat tails. The usefulness of collagen as a growth surface is also demonstrated by the availability of collagen-coated microcarrier beads (Cytodex-3, Pharmacia).

3.2.1 Surfaces for cell attachment
i. *Glass*

Alum-borosilicate glass (e.g., Pyrex) is preferred because soda-lime glass releases alkali into the medium and needs to be detoxified (by boiling in weak acid) before use. After repeated use glassware can become less efficient for cell attachment, but efficiency can be regained by treatment with 1 mM magnesium acetate. After several hours soaking at room temperature the acetate is poured away and the glassware is rinsed with distilled water and autoclaved.

ii. *Plastics*
Polystyrene is the most used plastic for cell culture, but polyethylene, polycarbonate, Perspex, PVC, Teflon, cellophane, and cellulose acetate are all suitable when pre-treated correctly.

iii. *Metals*

Stainless steel and titanium are both suitable for cell growth because they are relatively chemically inert, but have a suitable high negative energy. There are many grades of stainless steel, and care has to be taken in choosing those which do not leak toxic metallic ions. The most common grade to use for culture applications is 316, but 321 and 304 may also be

suitable. Stainless steel should be acid washed (10% nitric acid, 3.5% hydrofluoric-acid, 86.5% water) to remove surface impurities and inclusions acquired during cutting.

3.3 Scaling-up
3.3.1 Step 1: roller bottle

The aims of scaling-up are to maximize the available surface area for growth and to minimize the volume of medium and headspace, while optimizing cell numbers and productivity. Stationary cultures have only one surface available for attachment and growth, and consequently they need a large medium volume. The medium volume can be reduced by rocking the culture or, more usually, by rolling a cylindrical vessel. The roller bottle has nearly all its internal surface available for cell growth, although only 15-20% is covered by medium at any one time. Plastic disposable bottles are available in *c.* 750 cm^2 and *c.* 1500 cm^2 (1400-1750 cm^2) sizes. Rotation of the bottle subjects the cells to medium and air alternately, as compared with the near anaerobic conditions in a stationary culture. This method reduces the volume of medium required, but still requires a considerable headspace volume to maintain adequate oxygen and pH levels. The scale-up of a roller bottle requires that the diameter is kept as small as possible. The surface area can be doubled by doubling the diameter or the length. The first option increases the volume (medium and headspace) fourfold, the second option only twofold.

The only means of increasing the productivity of a roller bottle and decreasing its volume is by using a perfusion system. This is an expensive option, as an intricate revolving connection has to be made for the supply lines to pass into the bottle. However, cell yields are considerably increased and extensive multi-layering takes place.

3.3.2 Step 2: roller bottle modifications

The roller bottle system is still a multiple process, and thus inefficient in terms of staff resources and materials. To increase the surface area within the volume of a roller bottle, the following vessels have been developed (Figure 3).

i. SpiraCel
Bibby Sterilin have replaced their bulk cell culture vessel with a SpiraCel roller bottle. This is available with a spiral polystyrene cartridge in three sizes. 3000, 4500, and 6000 cm^2. It is crucial to get an even distribution of the cell inoculums throughout the spiral, otherwise very uneven growth and low yields are achieved. Cell growth can be visualized only on the outside of the spiral, and this can be misleading if the cell distribution is uneven,

ii. Glass tubes
A small scale example is the Bellco-Corbeil Culture System (Bellco), A roller bottle is packed with a parallel cluster of small glass tubes (separated by siliconc spacer rings). Three versions are available giving surface areas of 5 x 10^3, 1 x 10^4, and 1.5 x 10^4 cm^2. Medium is perfused through the vessel from a reservoir. The method is ingenious in that it alternately rotates the bottle 360° clockwise and then 360° anticlockwise. This avoids the use of special caps for the supply of perfused medium. An example of its use is the production of 3.2 X 10^9 Vero cells (2.3 X 10^5/cm^2) over six days using 6.5 litres of medium (perfused at 50 ml/min) in the 10000 cm^2 version.

iii. Increased surface area roller bottles

In place of the smooth surface in standard roller bottles the surface is 'corrugated', thus doubling the surface area within the same bottle dimensions; e.g., extended surface area roller bottle (ESRB) available from Bibby Sterilin (Corning), or the ImmobaSil surface which is a textured silicone rubber matrix surface (Ashby Scientific Ltd. or Integra Biosciences).

3.3.3 Step 3: large capacity stationary cultures
i. *Cell factory (multitray unit)*

The standard cell factory unit (Nunc) comprises ten chambers, each having a surface area of 600 cm^2, fixed together vertically and supplied with interconnecting channels. This enables all operations to be carried out once only for all chambers. It can thus be thought of as a flask with a 6000 cm^2 surface area using 2 litres of medium and taking up a total volume of 12 500 ml. In practice this unit is convenient to use and produces good results, similar to plastic flasks. It is made of tissue culture grade polystyrene and is disposable. One of the disadvantages of the system can be turned to good use. In practice it is difficult to wash out all the cells after harvesting with trypsin, etc. However, enough cells remain to inoculate a new culture when fresh medium is added. Given good aseptic technique, this disposable unit can be used repeatedly. The system is used commercially for interferon production (by linking together multiples of these units). In addition, units are available giving 1200 (2 tray) and 24 000 cm^2 (40 tray).

ii. *Costar CellCube*

The CellCube has parallel polystyrene trays in a modular closed-loop perfusion system with an oxygenator, pumps, and a system controller (pH, O_2, level control). The unit is compact, with the trays being only 1 mm apart; thus the smallest unit of 21250 cm^2 is less than 5 litres total volume (1.25 litres medium). Additional units of 42500 cm^2 (2.5 litres medium) and 85000 cm^2 (5 litres medium) are available and four units can be run in parallel, giving 340 000 cm^2 growth area.

iii. *Hollow fibre culture*

Bundles of synthetic hollow fibres offer a matrix analogous to the vascular system, and allow cells to grow in tissue-like densities. Hollow fibres are usually used in ultrafiltration, selectively allowing passage of macromolecules through the spongy fibre wall while allowing a continuous flow of liquid through the lumen. When these fibres are enclosed in a cartridge and encapsulated at both ends, medium can be pumped in and will then perfuse through the fibre walls, which provide a large surface area for cell attachment and growth. Culture chambers based on this principle are available from Amicon. The capillary fibres, which are made of acrylic polymer, are 350 um in diameters with 75 um walls. The pores through the internal lumen lining are available with molecular mass cut-offs between 10000 and 100000. It is difficult to calculate the total surface area available for growth but units are available in various sizes and these give a very high ratio of surface area to culture volume (in the region of 30 cm^2/ml). Upto 10^8 cells/ml has been maintained in this system. These cultures are mainly used for suspension cells but are suitable for attached cells if the polysulfone type is used.

iv. Opticell culture system

This system (Cellex Biosciences Inc.) consists of a cylindrical ceramic cartridge (available in surface area between 0.4-12.5 m^2) with 1 mm^2 square channels running lengthways through the unit. A medium perfusion loop to a reservoir, in which environmental control is carried out, completes the system. It provides a large surface area/volume ratio (40:1) and its suitability for virus, cell surface antigen, and monoclonal antibody production is documented. Scale-up to 210 m^2 is possible with multiple cartridges arranged in parallel in a single controlled unit. Cartridges are available for both attached and suspension cells, which become entrapped in the rough porous ceramic texture.

v. Heli-Cel (Bibby Sterilin)

Twisted helical ribbons of polystyrene (3 mm x 5-10 mm x 100 um) are used as packing material for the cultivation of anchorage-dependent cells. Medium is circulated through the bed by a pump, and the helical shape provides good hydrodynamic flow. The ribbons are transparent and therefore allow cell examination after removal from the bed.

3.3.4 Step 4: unit process systems

There are basically three systems which fit into fermentation (suspension culture) apparatus (Figure 3):

- cells stationary, medium moves (e.g., glass bead reactor)
- heterogeneous mixing (e.g., stack plate reactor)
- homogeneous mixing (e.g., microcarrier)

i. Bead bed reactor

The use of a packed bed of 3-5 mm glass beads, through which medium is continually perfused has been reported by a number of investigators since 1962. The potential of the system for scale-up uses a 100 litre capacity system for the growth of BHK21 cells and systems can be commercially obtained (Meredos GmbH).
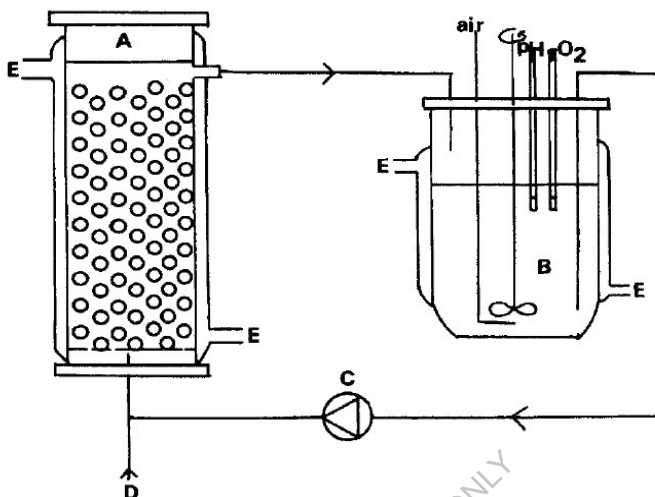
Figure 4. A glass bead bioreactor. (A) Glass bead bed; (B) reservoir; (C) pump; (D) inoculation and harvest line; (E) temperature-controlled water jackets.

Spheres of 3 mm diameter pack sufficiently tightly to prevent the packed bed from shifting, but allow sufficient flow of medium through the column so that fast flow rates, which would cause mechanical shear damage, are not needed. The physical properties of glass spheres are given in Table 3 and these data allow the necessary culture parameters to be calculated. A system which can be constructed in the laboratory is illustrated in Figure 4. Medium is transferred by a peristaltic pump in this example, but an airlift driven system is also suitable and gives better oxygenation. Medium can be passed either up or down the column with no apparent difference in results.

Table 3. Properties of 3 mm and 5 mm glass beads

Glass sphere diameter	3mm	5mm
Weight/g	0.0375	0.0375
Surface area (cm^2/kg)	7546	4570
Volume (ml/kg)	990	625
Void fraction	0.46	0.4
Void volume (ml/kg)	455	250
Channel size (mm2)	0.32	1
Vero cell yields		
106/cm^2	0.78	2.5/3.1
108/kg	4	8.0/9.9
106/ml	1.6	3.2/4.0

• Yield in 1 kg/10 kg bed reactor

Although 3 mm beads arc normally used to maximize the available surface area, the use of 5 mm spheres actually gives a higher total tell yield despite the reduced surface area per unit volume. A development of this system (porous beads) which significantly increases cell yields.

ii. *Heterogeneous reactor*

Circular glass or stainless steel plates are fitted vertically, 5-7 mm apart, on a central shaft. This shaft may be stationary, with an airlift pump for mixing, or revolving around a vertical (6 r.p.h.) or horizontal (50-100 r.p.m.) axis. This multisurface propagator (20) was used at sizes ranging from 7.5-200 litres, giving a surface area of up to $2 \times 10^5/cm^2$. The author's experience is solely with the horizontal stirred plate type of vessel (Figure 3, stack plate), which is easier to use and has been successful for both heteroploid and human diploid cells. The main disadvantage with this type of culture is the high ratio of medium volume to surface area (1 ml to 1-2 cm^2). This cannot be altered with the horizontal types, although it can be halved with the vertically revolving discs.

iii. *Homogeneous systems (microcarrier)*

When cells are grown on small spherical carriers they can be treated as a suspension culture, and advanced fermentation technology processes and apparatus can be utilized. The method uses dextran beads (Sephadex A-50). These were not entirely satisfactory, because the charge of the beads was unsuitable, and possibly due to toxic effects. However, much developmental work has since resulted in many suitable microcarriers being commercially available (Table 4). The choice of this microcarrier was based on a preference for a dried product which could be accurately weighed and then prepared *in situ,* and the fact that with a density of only 1.03 g/ml this product could be used at concentrations of up to 15 g/litre (90 000 cm^2/litre). However, this preference does not detract from the quality of other microcarriers, most of which have been used with equal success.

Culture apparatus

A spinner vessel is not suitable unless the stirring system is modified. Paddles with large surface area are needed. These are commercially available but can be easily constructed out of a silicone rubber sheet and attached to the magnet with plastic ties. The advantage of constructing one within the laboratory is that the blades can be made to incline 20-30° from the vertical, thus giving much greater lift and mixing than vertical blades (Figure 5). Microcarrier cultures are stirred very slowly (maximum 75 r.p.m.) and it is essential to have a good quality magnetic stirrer that is capable of giving a smooth stirring action in the range of 20-100 r.p.m. Never use a stirrer mechanism that has moving surfaces in contact with each other in the medium; otherwise the microcarriers will be crushed. Thus, stirrers which revolve on the bottom of the vessel are unsuitable. As mixing, and thus mass transfer, is so poor in these cultures, the depth of medium should not exceed the diameter by more than a factor of two unless oxygenation systems or regular medium changes are performed. In the basic culture systems, medium changes have to be carried out at frequent intervals. It is worthwhile, therefore, to make suitable connections to the culture vessel to enable this to be done conveniently *in situ.* This will pay dividends in reducing the chances of contaminating the culture. A simplified culture set-up is shown in Figure 6.

Table 4. Comparison of microcarriers

Trade name	Manufacturer gravity	Material (urn)	Specific (cm²/g)	Diameter	Area
Biosilon	Nunc	Polystyrene	1.05	160-300	255
Bioglas	Solohill Eng.	Glass[a]	1.03	150-210	350
Bioplas	Solohill Eng.	Polystyrene[a]	1.04	150-210	350
Biospheres	Solohill Eng.	Collagen[a]	1.02	150-210	350
Cytodex-1	Pharmacia	DEAE Sephadex	1.03	160-230	6000
Cytodex-2	Pharmacia	DEAE Sephadex	1.04	115-200	5500
Cytodex-3	Pharmacia	Collagen	1.04	130-210	4600
Cytosphere	Lux	Polystyrene	1.04	160-230	250
Dormacell	Pfeifer & Langen	Dextran	1.05	140-240	7000
DE-53	Whatman	Cellulose	1.03	Fibres	4000
Gelibead	Hazelton Lab.	Gelatin	1.04	115-235	3800
Microdex	Dextran Prod.	DEAE dextran	1.03	150	250
Ventreglas	Ventrex	Glass	1.03	90-210	300
Ventregel	Ventrex	Gelatin	1.03	150-250	4300

Biospheres (glass, plastic, collagen) available at specific gravity of 1.02 or 1.04 and diameters of 150-210 or 90-150 um (manufactured by Solohill Eng. and distributed by Whatman and Cellon)
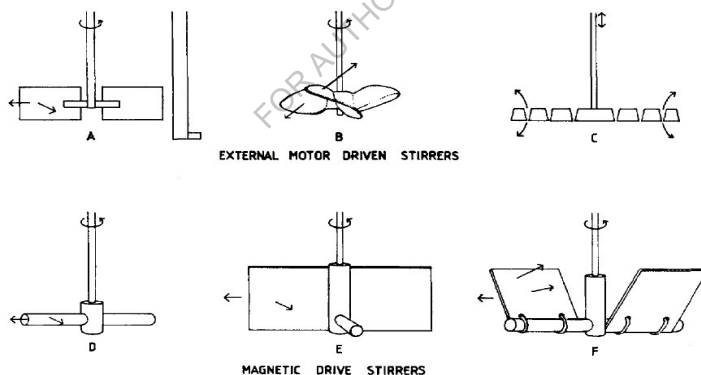
Figure 5. Types of impellers for growing suspension and microcarrier cells (A) Flat disc turbine, d2:dl = 0.33, radial flow, turbulent mixing; (B) marine impeller, d2:dl = 0.33, axial flow, turbulent mixing, blade angle 25°; (C) Vibro-mixer; (D) stirrer bar, d2:dl = 0.6, radial flow, laminar mixing; (E) vertical and (F) angled (25°) paddle, dl:d2 = 0.6-0.9, axial and radial flow, laminar and turbulent mixing. (d1 is the diameter of the vessel and d2 is the diameter of the impeller.)
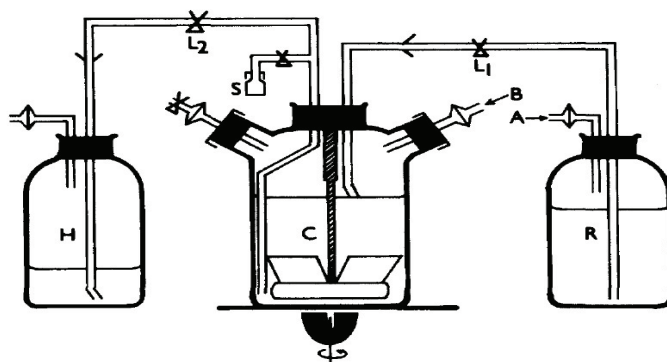
Figure 6. A simple microcarrier culture system allowing easy medium changing. To fill the culture (C) open clamp (L1) and push the medium from the reservoir (R) using air pressure (at A). To harvest, stop the stirring for 5 min, open L2, and push the medium from (C) to (H) using air pressure (at B). (S) is a sampling point.

Initiation of the culture

Many of the factors are critical when initiating a microcarrier culture. Microcarriers are spherical, and cells will always attach to an area of minimum curvature. Therefore a microcarrier surface can never be ideal, however suitable its chemical and physical properties. Ensure the medium and beads are at a stable pH and temperature, and inoculate the cells (from a logarithmic, not a stationary culture) into a third of the final medium volume. This increases the chances of cells coming into contact with the microcarriers. Microcarrier concentrations of 2-3 g/litre should be used. Higher concentrations need environmental control or very frequent medium changes.

After the attachment period (3-8 h), slowly top up the culture to the working volume and increase the stirring rate to maintain completely homogeneous mixing. If these conditions are adhered to, and there are no changes in temperature and pH, then all cells that grow on plastic surfaces should readily initiate a microcarrier culture.

Maintenance of the **culture**

It is very easy to monitor the progress of cell growth in a microcarrier culture. Samples can be easily removed, cell counts (by nuclei counting) and glucose determinations carried out, and the cell morphology examined. As the cells grow, so the beads become heavier and need an increased stirring rate. After three days or so the culture will become acidic and need a medium change. Again, this is an extremely easy routine: turn off the stirrer, allow the beads to settle for 5 min, and decant off as much medium as desired. Top up gently with fresh medium (pre-warmed to 37°C) and restart the stirring.

Harvesting

It is very difficult to harvest many cell types from microcarriers unless the cell density on the bead is very high, and cells do not multilayer on microcarriers to the same degree as in stationary cultures. Harvesting can be attempted by draining off the medium, washing the beads at least once in buffer, and adding the desired enzyme. Stir the culture fairly rapidly (75-125 r.p.m.) for 20-30 min. If the cells detach, a high proportion can be collected by allowing the beads to settle out for 2 min and then decanting off the supernatant. For a total harvest pour the mixture into a sterilized sintered glass funnel (porosity grade 1). The cells will pass through the filter, but the microcarriers will not. Alternatively, insert a similar filter (usually stainless steel) into the culture vessel. If the microcarrier is dissolved then the cells can be released into suspension completely undamaged, and therefore of better quality than when the cells are trypsinized. Usually this is a tar quicker method than trypsinization of cells. Gelatin beads are solubilized with trypsin and/or EDTA, collagenase acts on the collagen-coated beads, and dextranase can be used on the dextran microcarriers.

Scaling-up of microcarrier culture

Scaling-up can be achieved by increasing the microcarrier concentration, or by increasing the culture size. In the first case nutrients and oxygen are very rapidly depleted, and the pH falls to non-physiological levels. Medium changes are not only tedious but provide rapidly changing environmental conditions. Perfusion, either to waste or by a closed-loop, must be used to achieve cultures with high microcarrier concentration. This can be brought about only by an efficient filtration system so that medium without cells and microcarriers can be withdrawn at a rapid rate (Figure 7). This is constructed of a stainless steel mesh with an absolute pore size in the 60-120 µm range. Attachment to the starrer shaft means that a large surface area filter can be used and the revolving action discourages cell attachment and clogging. However scaling-up is achieved, oxygen limitation is the chief factor to overcome. This is an especially difficult problem in microcarrier culture because stirring speeds are low. Sparging cannot be used as microcarriers get left above the medium level due to the foaming it causes. The perfusion filter previously described, however, does allow sparging into that part of the culture in which no beads are present. Unfortunately, this means that the most oxygenated medium in the culture is removed by perfusion, but at a sparging rate of 10 cm^3 of oxygen/litre considerable diffusion of oxygenated medium occurs into the main culture. However, modified spin filters are available which have a separate compartment for perfusion and sparging. Thus oxygen delivery to a microcarrier culture can utilize the following systems (see also Table 2):

I.   Surface aeration.
II.   Increasing the perfusion rate of fully oxygenated medium from the reservoir.
III. Sparging into the filter compartment.

These three systems are used by the author in the system illustrated in Figure *7* to run cultures at up to 15 g/litre Cytodex-3 in culture vessels between 2-20 litre working volumes. A typical experiment is shown in Figure 8.
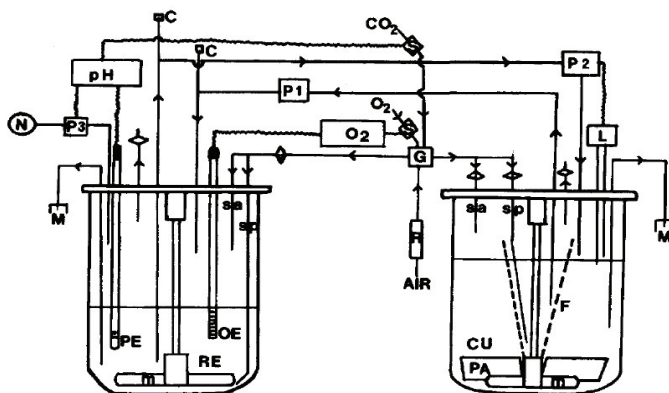
Figure 7. A closed-loop perfusion system with environmental control to allow high cell density microcarrier culture. (CU) Culture vessel; (RE) reservoir; (C) connector for medium changes and harvesting; (F) filter; (G) gas blender; (L) level controller; (M) sampling device; (m) magnet; (N) alkali (NaOH) reservoir; (OE) oxygen electrode; (PA) paddle; (PE) pH electrode; (P1-P3) pumps: 1, medium to reservoir (continuous), 2, medium to culture (controlled by L), 3, alkali to reservoir (controlled by PE); (R) rotameter measuring gas flow rate; (sa) gas supply for surface aeration; (sp) gas supply for sparging; (S) solenoid valve.
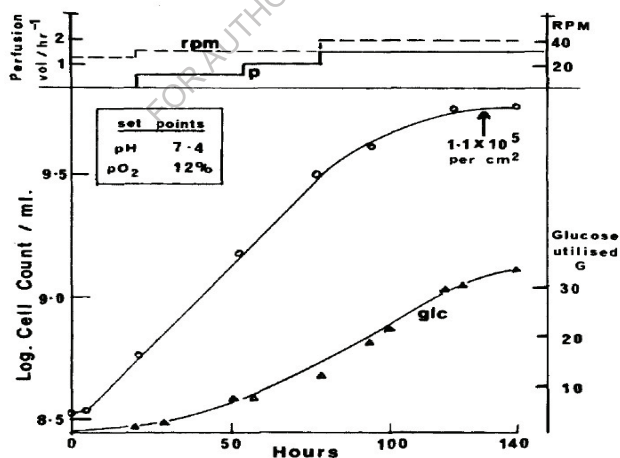
Figure 8. Growth of GPK epithelial cells on Cytodex-3 (10 g/litre) in the 10 litre culture system described in *Figure 7.* (o-o) Cell concentration; (A-A) glucose (glc) utilized; (p) perfusion rate.

Summary of microcarrier culture

Microcarrier cultures are used commercially for vaccine and interferon production in fermenters up to 4000 litres. These processes use heteroploid or primary cells. One of the problems of using very large scale cultures is that the required seed inoculum gets progressively larger. Harvesting of cells from large unit scale cultures is not always very successful, although the availability of collagen and gelatin microcarriers eases this problem considerably. In *situ* trypsinization of cells is the usual method of choice, but it is difficult to remove a high percentage of the cells in a viable condition. Filters incorporated into the culture vessel provide the best solution and are most efficient with the 'hard' (glass, plastic) microcarriers which do not block the filter pores like the dextran and gelatin microcarriers. Some microcarriers can be washed and reused but this, of course, is not possible with the gelatin- or collagenase-treated beads.

3.3.5 Summary: choice of equipment

A wide range of commercially available and laboratory-made equipment has been reviewed. This should enable a choice to be made, depending upon the amount and type of cells or product needed and the financial and staff resources available. To review the choices available, Figure 3 and Table 5 should be consulted.

4 Suspension culture

As indicated previously in this chapter, suspension culture is the preferred method for scaling-up cell cultures. Some cells, especially those of haemopoietic derivation, grow best in suspension culture. Others, particularly transformed cells, can be adapted or selected, but some, for example human diploid cell strains (W1–38, MRC-5), will not survive in suspension at all. A further factor that dictates whether suspension systems can be used is that some cellular products are expressed only when the cell exists in a monolayer or if cell-to-cell contact is established (e.g., for the spread of intracellular viruses through a cell population).

4.1 Adaptation to suspension culture

Cell lines vary in the ease with which they can be persuaded to grow in suspension. For those that have the potential, there are two basic procedures that can be used to generate a suspension cell line from an anchorage-dependent one.

4.1.1 Selection

This method, as demonstrated by the derivation of the LS cell line from L-929 cells and the HeLa-S3 clone from HeLa cells, depends on the persistence of loosely attached variants within the population. A confluent monolayer culture is lightly tapped or gently swirled, the medium decanted from the culture, and cells in suspension recovered by centrifugation. Cells have to be collected from many cultures to provide a sufficient number to start a new culture at the required inoculum density (at least 2×10^5/ml), This procedure has to be repeated many times over a long period, because many of the cells that are collected are only in the mitotic phase, rather than potential suspension cells (cells round up and become very loosely attached to the substrate during mitosis). Eventually it is possible with some cell lines to derive a

viable cell population that divides and grows in static suspension, just resting on the substrate rather than attaching and spreading out.

4.1.2 Adaptation

This method probably works the same way as the selection procedure, except that a selection pressure is exerted on the culture while it is maintained in suspension mechanically. However, with many cell lines the relatively large number that becomes anchorage-independent suggests it is more than just selection of a few variant cells.

Table 5. Comparison of monolayer culture systems, showing their relative productivity and scale-up potential

Culture	Unit surface area (cm^2)	Surface area: medium ratio[a] (litre)	Max. vol
Multiple processes:			
Roux bottle	175-200	1-2	
Roller bottle	850-175	3	
-Corrugated	1700	6	
-Spiracell	6000	6	
-Corbeil	15 000	2.5	
Cell Factory	24000	2.5	
Hollow fibres	18000	72	
Opticell	120000	30	
CellCube	340000	17	
Unit processes:			
Heli-Cel	16 000/L	16	(l)[b]
Glass spheres	7500/L	18	200
Porous glass spheres	80 000/L	150	(10)[b]
Stack plates	1250/L	1.25	200
Microcarrier (5 g/litre)	35000/L	35	4000
Porous microcarrier	300 000/L	300	(24)[b]

[a]Volume in bioreactor-does not allow for media changes or reservoir.
[b]Current experimental scales-scale-up potential.

It is usually necessary to maintain the newly established cell strain in stirred suspension culture, because reversion to anchorage dependence can occur in static cultures. Sometimes cells will adhere to the substrate without becoming completely spread out and continue to grow and divide with a near-spherical morphology.

4.2 Static suspension culture

Many cell lines will grow as a suspension in a culture system used for monolayer cells (i.e., with no agitation or stirring). Cell lines that are capable of this form of growth include the many lymphoblast lines (e.g., MOLT, RAJI), hybridomas, and some non-haemopoietic lines, such as the LS cells described in the previous section. However, with the latter type of cells there is always the danger of reversion to a monolayer (e.g., a small proportion of the LS cell line always attaches and is discarded at each subcultivation). Static suspension culture is unsuitable for scale-up, for reasons already stated for monolayer culture.

4.3 Small scale suspension culture

Small scale means less than 2 litres. This may seem an entirely arbitrary definition, but it is made on the basis that above this volume additional factors apply. The conventional laboratory suspension culture is the spinner flask, so-called because it contains a magnetic bar as the stiirer, and this is driven from below the vessel by a revolving magnet. Details of some of the readily available stirrer vessels, together with suppliers and available size range, are given in Figure 9.

The range of options is:
a. Conventional vessels with spin bar (Bellco, Wheaton).
b. Radial (pendulum) stirring system (figure 10) for improved mixing at low speeds (under 100 r.p.m.) (Techne. Integra Biosciences CellSpin).
c. Bellco dual overhead drive system (radial stirring and permits perfnsion).
d. Techne Br-06 floating impeller system allows working volumes of 500 ml to 3 litres to be used and increased during culture.

Note: if clumping or attachment to the culture vessel should occur the vessels can be siliconized with Dow Corning 1107 or Repelcotc (dimethyldichlorosilane) (Hopkins and Williams), or medium with reduced Ca^{2-} and Mg^{2+} can be used.
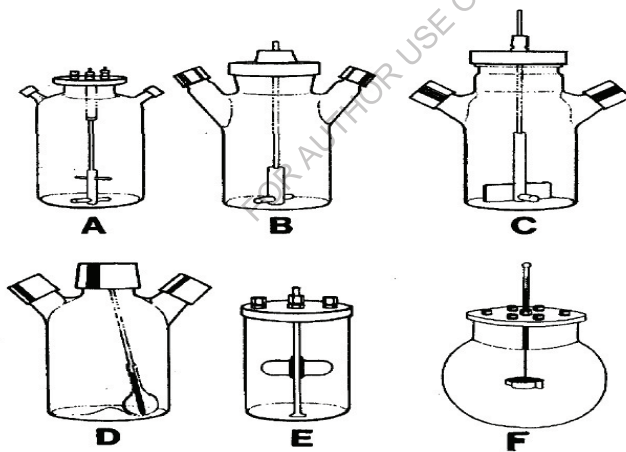
Figure 9. Commercially available spinner cultures. (A) LH Fermentation Biocul (1-20 litres); (B) Bellco and Wheaton Spinner Flasks (25 ml-2 litres); (C) Bellco and Cellon spinner (25 ml-2 litres); (D) Techne (25 ml-5 litres); (E) Techne Cytostat (1 litre); (F) Techne Br-06 Bioreactor (3 litres).
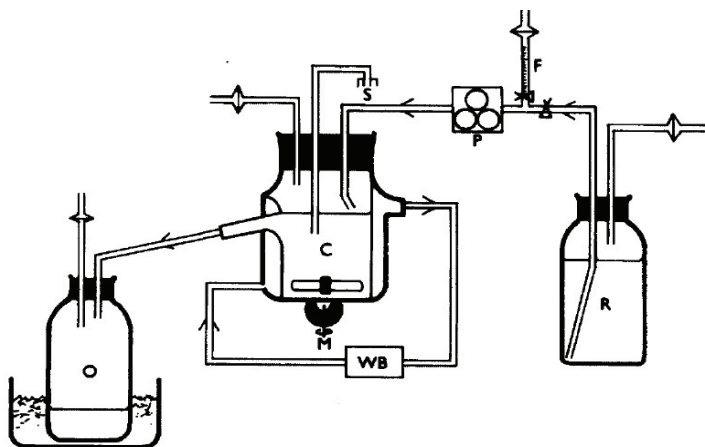
Figure 10. Continuous-flow culture system. (C) Water-jacketed culture vessel; (WB) water-bath and circulating system; (0) overflow vessel; (R) reservoir; (P) pump; (S) sampling device; (F) burette for measuring flow rate; (M) magnetic drive.

4.3.1 SuperSpinner

The SuperSpinner was developed to increase the productivity within a spinner flask without going to the complexity of the hollow fibre and membrane systems described below. It consists of a 1 litre Duran flask (Schott) equipped with a tumbling membrane stirrer moving a polypropylene hollow fibre through the medium to improve the oxygen supply. The small device is placed in a CO_2 incubator and a small membrane pump is connected via a sterilizing filter with the membrane (stirrer). The culture is placed on a magnetic stirrer and has a working volume from 300 ml to 1000 ml. Three different membrane lengths (1, 1.5, and 2 m) are available depending upon the degree of oxygenation the cell line needs. Cell concentrations in excess of 2×10^6/ml are achieved with mAb production of 160 mg/litre (specific productivity of mAb c. 50 Ug/10^6 cells). Repeated batch culture (80% media replacement every two to three days) over 32 days produced 970 mg mAb. This relatively inexpensive modification of a spinner culture is available from Braun Biotech.

4.4 Scaling-up factors

In scale-up, both the physical and chemical requirements of cells have to be satisfied. The chemical factors require environmental monitoring and control to keep the cells in the proper physiological environment. These factors include oxygen, pH, medium components, and removal of waste products. Physical parameters include the configuration of the bioreactor and the power supplied to it. The function of the stirrer impeller is to convert energy (measured as kW/m^3) into hydrodynamic motion in three dimensions (axial, radial, and tangential). The impeller has to circulate the whole liquid volume and to generate turbulence (i.e., it has to pump and to mix) to create a homogeneous blend, to keep cells in suspension, to optimize mass transfer rates between the different phases of the system (biological, liquid, and gaseous), and to facilitate heat transfer. Good mixing becomes increasingly difficult with scaling-up, and the power needed to attain homogeneity can cause

problems. The energy generated at the tip of the stirrer blade is a limiting factor as it gives rise to a damaging shear force. Shear forces are created by fluctuating liquid velocities in turbulent areas. The factors which affect this are: impeller shape (this dictates the primary induced flow direction) (Figure 5), the ratio of impeller to vessel diameter, and the impeller tip speed (a function of rotation rate and diameter). The greater the turbulence the more efficient the mixing, but a compromise has to be reached so that cells are not damaged. Large impellers running at low speeds give a low shear force and high pumping capacity, whereas smaller impellers need high stirring speeds and have high shear effects. Magnetic bar stirring gives only radial mixing and no lift or turbulence. The marine impeller is more effective for cells than the flat-blade turbine impellers found in many bacterial systems, as it gives better mixing at low stirring speeds. If the cells are too fragile for stirring, or if sufficient mixing cannot be obtained without causing unacceptable shear rates, then an alternative system may have to be used. Pneumatic energy, for example mixing air bubbles (e.g., airlift fermenter) or hydraulic energy (e.g., medium perfusion), can be scaled-up without proportionally increasing the power. To improve the efficiency of mechanical stirring, the design of the stirrer paddle can be altered (e.g., as described for microcarrier culture), or multiple impellers can be used.

A totally different stirring concept is the Vibro-mixer. This is a non-rotating agitator which produces a stirring effect by a vertical reciprocating motion with a path of 0.1-3 mm at a frequency of 50 Hz. The mixing disc is fixed horizontally to the agitator shaft, and conical shaped holes in the disc create a pumping action to occur as the shaft vibrates up and down. The shaft is driven by a motor which operates through an elastic diaphragm; this also provides a seal at the top of the culture. A fermentation system using this principle is available commercially (Vibro-Fermenter, Chemap). The advantages of this system are the greatly reduced shear forces, random mixing, reduced foaming, and reduced energy requirement, especially for scaling-up.

The significant effect on vessel design of moving from a magnetic stirrer to a direct drive system is the fact that the drive has to pass through the culture vessel. This means some complexity of design to ensure a perfect aseptic seal while transferring the drive, complete with lubricated bearings, through the bottom or top plate of the vessel. Culture vessels with magnetic coupling are becoming available at increasingly higher volumes, and overcome this problem of aseptic seals.

Scaling-up cannot be proportional; one cannot convert a 1 litre reactor into a 1000 litre reactor simply by increasing all dimensions by the same amount. The reasons for this are mathematical: doubling the diameter increases the volume threefold, and this affects the different physical parameters in different ways. One factor to be taken into consideration is the height/diameter ratio, as this is one of the most important fermenter design parameters. In sparged systems the taller the fermenter in relation to its diameter the better, as the air pressure will be higher at the bottom (increasing the oxygen solubility) and the residence time of bubbles longer. However, in non-sparged systems which are often used for animal cells and rely on surface aeration, the surface area: height ratio is more important and a 2:1 ratio should not be exceeded (preferably 1:1.5). Mass transfer between the culture phases has been discussed in relation to oxygen. It is this characteristic of scaling-up that demands the extra sophistication in culture design to maintain a physiologically correct environment. This sophistication includes impeller design, oxygen delivery systems, vessel geometry, perfusion loops, or a completely different concept in culture design to the stirred bioreactor.

4.5 Stirred bioreactors

The move from externally driven magnetic spinner vessels to fermenters capable of scale-up from 1 litre to 1000 litres and beyond, has the following consequences at some stage:

(a) Change from glass to stainless steel vessels.

(b) Change from a mobile to a static system: connection to steam for *in* situ sterilization; requirement for water jacket or internal temperature control; need for a seed vessel, a medium holding vessel, and downstream processing capability.

(c) Greater sophistication in environmental control systems to meet the increasing mass transfer equipment.

In practice, the maximum size for a spinner vessel is 10 litres. Above this size there are difficulties in handling and autoclaving, as well as the difficulty of being able to agitate the culture adequately. Fermenters with motor driven stirrers are available from 500 ml, but these are chiefly for bacterial growth. It is a significant step to move above the 10-20 litre scale as the cost of the equipment is significant (e.g., $40-50000 for a complete 35 litre system) and suitable laboratory facilities are required (steam, drainage, etc.). There is a wide range of vessels available from the various fermenter suppliers which include some of the following modifications:

- suitable impeller (e.g., marine)
- no baffles
- curved bottom for better mixing at low speeds
- water jacket rather than immersion heater type temperature control (to avoid localized heating at low stirring speeds)
- top-driven stirrer so that cells cannot become entangled between moving parts
- mirror internal finishes to reduce mechanical damage and cell attachment

Since adequate mixing, and thus mass transfer of oxygen into the vessel, can be maintained without damaging the cells, there is no maximum to the scale-up potential. Namalva cells have been grown in 8000 litre vessels for the production of interferon. There are many heteroploid cell lines, such as Vero, HeLa, and hybridomas, which would grow in such systems. Whilst regulatory agencies require pharmaceutical products to be manufactured predominantly from normal diploid cells (which will not grow in suspension), the only motive for using this scale of culture was for veterinary products. However, the licensing of products such as tPA, EPO, interferon, and therapeutic monoclonal antibodies from heteroploid lines means that large scale bioreactors are in widespread use. There are many applications for research products from various types of cells, and this is served by the 2-50 litre range of vessels. At present, the greatest incentive for large scale systems is to grow hybridoma cells (producing monoclonal antibody). In tissue culture the antibody yield is 50- to 100-fold lower than when the cells are passaged through the peritoneal cavity of mice, although the purity is greater, particularly if serumfree medium is used. The main need is to supply antibodies to meet the requirements for diagnostic and affinity chromatography purposes, but increasingly as new therapeutic and prophylactic drugs are being developed and licensed much larger quantities are required.

4.6 Continuous-flow culture
4.6.1 Introduction

At sub-maximal growth rates, the growth of a cell is determined by the concentration of a single growth-limiting nutrient. This is the basis of the chemostat, a fixed volume culture, in which medium is fed in at a constant rate, mixed with cells, and then leaves at the same rate. The culture begins as a batch culture while the inoculum grows to the maximum value that can be supported by the growth-limiting nutrient (assuming the dilution rate is less than the maximum growth rate). As the growth-limiting nutrient decreases in concentration, so the growth rate declines until it equals the dilution rate. When this occurs, the culture is defined as being in a 'steady state' as both the cell numbers and nutrient concentrations remain constant. When the culture is in a steady state, the cell growth rate (u) is equal to the dilution rate (D). The dilution rate is the quotient of the medium flow rate per unit time and the culture volume (V):

$$\mu = D = f/V \text{ day} \qquad \qquad 8$$

As the growth rate is dependent on the medium flow rate, the mean generation (doubling) time can be calculated:

$$\mu = \ln 2/D \qquad \qquad 9$$

An alternative system to the chemostat is the turbidostat in which the cell density is held at a fixed value by altering the medium supply. The cell density (turbidity) is usually measured through a photoelectric cell. When the value is below the fixed point, medium supply is stopped to allow the cells to increase in number. Above the fixed point, medium is supplied to wash out the excess cells. This system really works well only when the cell growth rate is near maximum. However, this is its main advantage over the chemostat, which is least efficient or controllable when operating at the cell's maximum growth rate.

4.6.2 Equipment

Complete chemostat systems can be purchased from dealers in fermentation equipment. However, systems can be easily constructed in the laboratory (Figure 10). The culture vessel needs a side-arm overflow at the required liquid level, which should be approximately half the volume of the vessel. If a suitable 37°C cabinet is not available, then a water-jacketed vessel is needed. Apart from this, all other components are standard laboratory items. Vessel enclosures can be made from silicone (or white rubber) bungs wired onto the culture vessel. A good quality peristaltic pump, such as the Watson-Marlow range, is recommended.

4.6.3 Experimental procedures

Recommended cells are LS, HeLa-S3, or an established lymphoblastic cell line such as L1210 or a hybridoma. Growth-limiting factors can be chosen from the amino acids (e.g., cystine) or glucose.

4.6.4 Uses

Continuous-flow culture provides a readily available continuous source of cells. Also, because optimal conditions or any desired physiological environment can be maintained, the culture is very suitable for product generation (as already shown for viruses and interferon).

For many purposes a two-stage chemostat is required so that optimal conditions can be met for cell growth (first stage) and product generation (second stage).

4.7 Airlift fermenter

The airlift fermenter relies on the bubble column principle both to agitate and to aerate a culture. Instead of mechanically stirring the cells, air bubbles are introduced into the bottom of the culture vessel. An inner (draft) tube is placed inside the vessel and mixing occurs because the air bubbles lift the medium (aerated medium has a lower density than non-aerated medium). The medium and cells which spill out from the top of the draft tube then circulate down the outside of the vessel. The amount of energy (compressed air) needed for the system is very low, shear forces are absent, and this method is thus ideal for fragile animal and plant cells. Also, as oxygen is continuously supplied to the culture, the large number of bubbles results in a high mass transfer rate. Culture units, as illustrated in Figure 11, are commercially available in sizes from 2-90 litres (Braun Biotech). The one disadvantage of the system is that scale-up is more or less linear (the 90 litre vessel requires nearly 4 m headroom). Whether it will be possible to use multiple draft tubes, and thus enable units with greatly increased diameters to be used, remains a developmental challenge. However, 2000 litre reactors are in operation for the production of monoclonal antibodies.

5 Immobilized cultures

Immobilized cultures are popular because they allow far higher unit cell densities to be achieved (50-200 x 10^6 cells/ml) and they also confer stability and therefore longevity, to cultures. Cells *in vivo* are in a three-dimensional tissue matrix; therefore immobilization can mimic this physiological state. Higher cell density is achieved by facilitating perfusion of suspension cells and increasing the unit surface area for attached cells. In addition many immobilization materials protect cells from shear forces created by medium flow dynamics. As cells increase in unit density they become less dependent on the external supply of many growth factors provided by serum, so real cost savings can be achieved. The emphasis has been on developing systems for suspension cells because commercial production of monoclonal antibody has been such a dominant factor. Basically two approaches have been used: immurement (confining cells within a medium-permeable barrier), and entrapment (enmeshing cells within an open matrix through which medium can flow unhindered). Immobilization techniques that can be scaled-up, such as the porous carriers will become a dominant production technology once the beads and process parameters have been optimized. They are currently excellent laboratory systems for the manufacture of cell products and, with gelatin carriers, production of cells.

5.1 Immurement cultures
5.1.1 Hollow fibres

These are very effective for suspension cells at scales up to about 1 litre (1-2 X 10^8 cells/ml). Simple systems can be set up in the laboratory by purchasing individual hollow fibre cartridges (e.g., Amicon, Microgon). All that is needed is a medium reservoir and a pump to circulate the medium through the intracapillary section of the cartridge, and a harvest line from the extracapillary compartment in which the cells and product reside. To get better performance there are a number of 'turn-key' systems that can be purchased, from relatively simple units, e.g., Kinetek, Cellco, Cell-Pharm, Amicon (Mini Flo-Path), Asahi Medical Co (Cultureflo), to extremely complex and sophisticated units (e.g., Cellex Acusyst)

capable of producing up to 40 g of monoclonal antibody per month. The Acusyst (Endotronics, now Cellex Biosciences Inc.) system made the breakthrough by using pressure differentials to simulate *in vivo* arterial flow. The unit has a dual medium circuit, one passing through the lumen, the other through the extracapillary (cell) space. By cyclically alternating the pressure between the two circuits, medium is made to pass either into, or out of, the lumen. This allows a flushing of medium through the cell compartment and overcomes the gradient problem, and gives the possibility of concentrating the product for harvesting. There are a range of Acusyst units, the Acusyst MiniMax and Maximiser being the laboratory models using respectively 2-3 and 3-8 litres of medium per day, and capable of running continuously for 50-150 days (four to five months being a typical run length). There is an initial growth phase of 10-25 days depending upon inoculum size, medium composition, and cell line, and when a density of 1-2 x 10^{10} cells is reached (1.1 m^2 cartridge, e.g., Acusyst Maximiser) production starts and daily harvesting of mAb begins. Cells cannot be sampled during the culture so reliance is placed upon a number of process measurements made on a regular basis-on-line pH and oxygen; off-line glucose, lactate, and if necessary ammonia and LDH (for loss of viability) at least weekly.

The Tecnomouse is another hollow fibre bioreactor containing up to five flat culture cassettes containing hollow fibres surrounded by a silicone membrane that gives uniform oxygenation and. nutrient supply of the culture and ensures homogeneity within the culture. The system comprises a control unit, a gas and medium supply unit, and the five culture cassettes. The culture is initiated with cells from 3 x 225 cm^2 flasks (5 x 10^7) and grows to 5 x 10^8 cells. The continuous medium supply is controllable and programmable with typical flow rates (perfusion or recirculation) of 30 ml/h increasing to 70 ml/h in 5 ml steps. After six days harvesting can be started and repeated every two to three days for 30-70 days. Each cassette gives 7 ml culture with 2,5-5.5 mg/ml mAb (equivalent to an average of 10 mg/day). Thus about 10 litres will yield 400 mg mAb/month and 1.5 g/month using the five cassettes (data based on manufacturer's information).

5.1.2 Membrane culture systems (miniPERM)

There has been a number of membrane-based culture units developed, many based on dialysis tubing, and even available as large fermenters (Bioengineering AG Membrane Laboratory Fermenter with a Cuphron dialysis membrane of 10000 Dalton molecular weight cut-off forming an inner chamber). One of the more successful and currently available systems is described here -the miniPERM Bioreactor (Heraens Instruments).

The miniPERM Bioreactor consists of two components, the production module (40 ml) containing the cells and the nutrient module (600 ml of medium). The modules are separated by a semi-permeable dialysis membrane (MWCO 12.5 kDa) which retains the cells and mAb in the production module but allows metabolic waste products to diffuse out to the nutrient module. There is a permeable silicone rubber membrane for oxygenation and gas exchange in the production module. The whole unit rotates (up to 40 rp.m.) within a CO_2 incubator. It can be purchased as a complete disposable ready to use unit or the nutrient module (polycarbonate) can be autoclaved and reused at least ten times.

5.1.3 Encapsulation

The entrapment of cells in semi-solid matrices, or spheres, has many applications, but the basic function is to stabilize the cell and thus protect it from suboptimal conditions. Cells

can be immobilized by adsorption, covalent bonding, crosslinking, or entrapment in a polymeric matrix. Materials that can be used are gelatin, polylysine, alginate, and agarose; the choice largely depends upon the problem being addressed. The matrix allows free diffusion of nutrients and generated product between the enclosed microenvironment and the external medium, Alginate is a poly sac charide and is crosslinked with Ca^{2+} ions. The rate of crosslinking is dependent on the concentration of Ca^{2+} (e.g., 40 min with 10 mM CaCl2). A recommended technique is to suspend the cells in isotonic NaCl buffered with Tris (1 mM) and 4% sodium alginate, and to add this mixture dropwise into a stirred solution of isotonic NaCl, 1 mM Tris, 10 mM *Ca.* $^{2+}$ at pH 7.4. The resulting spheres are 2-3 mm in diameter. The entrapped cells can be harvested by dissolving the polymer in 0. 1 M EDTA or 35 mM sodium citrate. Disadvantages of alginate are that calcium must be present and phosphate absent and that large molecule, such as monoclonal antibodies, cannot diffuse out. For these reasons, agarose in a suspension of paraffin oil provides a more suitable alternative. *5% agarose in PBS free of Ca^{2+} and Mg^{2+} is melted at 70°C, cooled to 40°C, and mixed with cells suspended in their normal growth medium. This mixture is added to an equal volume of paraffin oil and emulsified with a Vibro-mixer. The emulsion is cooled in an ice-bath, growth medium added, and, after centrifugation, the oil is removed. The spheres (80-200 um) are washed in medium, centrifuged and, after removing the remaining oil, transferred to the culture vessel, A custom-made unit can be purchased (Bellco Bioreactor), This 3 litre fluidized bed culture (bubble column) actually encapsulates the cells in hydrogel beads within the culture vessel and comes with a range of modular units to control all process parameters.

5.2 Entrapment cultures
5.2.1 Opticell

The special ceramic cartridges for suspension cells (S Core) entrap the cells within the porous ceramic walls of the unit. They are available in sizes from 0.42 m^2 to 210 m^2 (multiple cartridges), which will support 5 x 10^{10} cells with a feed/harvest rate of 500 litres/day and give a yield of about 50 g of monoclonal antibody per day.

5.2.2 Fibres

A simple laboratory method is to enmesh cells in cellulose fibres (DEAE, TLC, QAE. TEAE; all from Sigma). The fibres are autoclaved at 30 mg/ml in PBS, washed twice in sterile PBS, and added to the medium at a final concentration of 3 mg/ml in a spinner stirred bioreactor. This method has even been found suitable for human diploid cells.

5.3 Porous carriers

Microcarriers and glass spheres are restricted to attached cells and, because a sphere has a low surface area/volume ratio, restricted in their cell density potential. Their advantages are summarized in Table 6. A change from a solid to porous sphere of open, interconnecting pores (Figure 12), increases their potential enormously. There are various types of porous microcarrier commercially available (Table 4) A characteristic of these porous carriers is their equal suitability for suspension tells (by entrapment) and anchorage-dependent cells (huge surface area).

The problem with many immobilization materials is that diffusion paths become too long, preventing scale-up, A sphere is ideal in that cells and nutrients have only to penetrate 30% of the diameter to occupy 70% of the total volume. This facilitates scale-up as each

sphere, whether in a stirred, fluidized, or fixed bed culture, can be considered an individual mini-bioreactor.

5.3.1 Fixed bed (Porosphere)

The apparatus and experimental procedures for solid spheres is equally suited to porous Siran spheres (Schott) of 4-6 mm diameter. The only differences are that the bed should be packed with ovendried spheres and the void volume of cells plus medium inoculated (2 x 10^6/ml) directly to the bed (dry beads permit better penetration of cells). A larger medium volume is required, and faster perfusion rates (5-20 linear cm/min) should be used (cells are protected from medium shear within the matrix), e.g., 1 litre packed bed needs at least a 15 litre reservoir. After the initial 72 h period, 10 litres of fresh medium is added daily.

This method is a low investment introduction to high productivity production of mAb which is simple to use and reliable with low maintenance, at least for the first 50 days of culture.
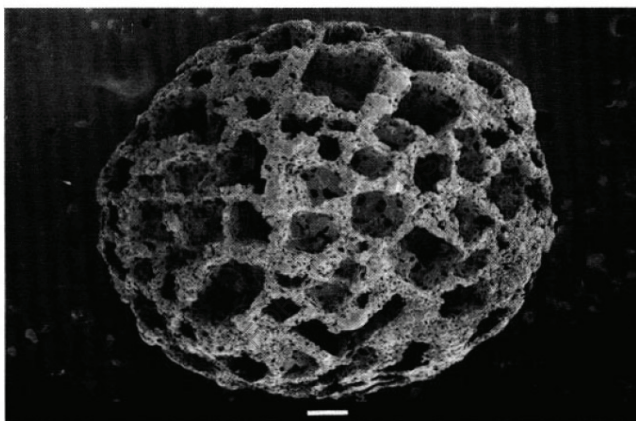
Figure 12. A Siran (Schott Glaswerke) porous glass sphere (120 xm diameter)

Table 6. Porous carriers—advantages compared to solid carriers
- Unit cell density 20- to 50-fold higher.
- Support both attached and suspension cells.
- Immobilization in 3D configuration easily achieved.
- Short diffusion paths into a sphere.
- Suitable for stirred, flttidized, or fixed bed reactors.
- Good scale-up potential by comparison with analogous systems (e.g., microcarrier at
- 4000 litre).
- Cells protected from shear.
- Capable of long-term continuous culture.

5.3.2 Fluidized beds

Porous microcarrier technology, currently the most successful scale-up method for high density perfused cultures, was pioneered by the Verax Corporation (now Cellex Biosciences Inc.). Turn-key units were available from 16 ml to 24 litres fluidized beds. The smallest system in the range, Verax System One, is a benchtop continuous perfusion fluidized bioreactor suitable for process assessment and development, and for laboratory scale production of mAbs. Cells are immobilized in porous collagen microspheres, weighted to give a specific gravity of 1.6, which allows high recycle flow rates (typically 75 cm/min) to give efficient fluidization. The microspheres have a sponge-like structure with a pore size of 20-40 um and internal pore volume of 85% allowing immobilization of cells to densities of 1-4 X 10^8/ml. They are fluidized in the form of slurry.

The Verax system comprises a bioreactor (fluidization tube), a control system (for pH, oxygen, medium flow rates), gas and heat exchanger and medium supply, and harvest vessels. The system is run continuously for long periods (typically over 100 days). In the author's laboratory it produced 15 x 10^{10} cells/litre and 540 mg mAb/litre/day (compared to166 in the fixed bed described above, 25.5 in a stirred reactor, and 18.5 mg in an airlift fermenter). In summary, it is probably the most productive system available, giving the cells a very high specific production rate, but does require some skill to operate to its maximum potential.

An alternative commercial system is the Cytopilot (Pharmacia Vogelbusch) which is a fluidized system using polyethylene carriers (Cytoline) and supports 12 X 10^7 cells/ml carriers. It is available as the Cytopilot-Mini (400 ml bed) for laboratory scale operation as well as sizes up to 25 litres. The unit has a magnetic stirrer that drives the medium up through a distribution plate into the upper chamber in which the microcarriers are lifted by hydrodynamic pressure. The degree of fluidization is controlled by the stirrer speed and a clear boundary layer is kept at the top of the culture so that clear medium can flow through the internal central circulation tube (loop) back to the stirrer. The culture is oxygenated by a mini-sparger delivering microbubbles and a medium feed rate of up to 25 bed matrix volumes per day giving high productivity. The unit can also be used as a packed bed bioreactor if the circulation system is reversed. There is a range of porous microcarriers available (Table 7) for fluidized systems. Alternatively, some of the carriers are designed for stirred cultures.

Table 7. Commercially available porous (micro) carriers

Trade name	Supplier	Material	Dlam (μm)	Culture mode[a]
Cultispher G	HyClone	Gelatin	170-270	F
			300-500	
Cytocell	Pharmacia	Cellulose	180-210	S
Cellsnow	Kirin Ltd.	Cellulose	800-1000	S
Cytoline 1, 2	Pharmacia	Polyethylene	1200-1500	F
ImmobaSil	Ashby Scientific	Silicone rubber	1000	S
Microsphere	Cellex Biosciences	Collagen	500-600	F
Si ran	Schott Glasswerke	Glass	600-1000	F
			4000-6000	X
Microcarrier	Asahi Chem. Ind.	Cellulose	300	S

[a]S, stirred; F, fluidized; X, fixed bed.

5.3.3 Stirred cultures

The Cultispher-G (gelatin), Cellsnow (cellulose), and ImmobaSil (silicone rubber) microcarriers are the most suited to stirred bioreactors, and can be used in an identical manner to solid microcarriers, i.e., 2 g/litre in shake flasks, spinner flasks, or stirred fermenters. The silicone rubber of the ImmobaSil microcarriers facilitates oxygen diffusion and this offers a great advantage over other formulations. About a 40-fold higher concentration of attached cells, and even greater densities of suspension cells, can be achieved over solid microcarriers. The cells can be released from the microcarriers by collagenase treatment.

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Chapter 4
Cell line preservation and authentication

1 Introduction

Thousands of different cell lines have been derived from human and other metazoan tissues. Many of these originate from normal tissues and exhibit a definable, limited doubling potential. Other cell lines may be propagated continuously, either having gone through an engineered or spontaneous genetic change from the normal primary population, or having been developed initially from tumour tissue. Both finite lines of sufficient doubling potential and continuous lines can be expanded to produce a large number of aliquots, frozen, and authenticated for widespread use in research.

The advantages of working with a well-defined cell line free from contaminating organisms would appear obvious. Unfortunately, however, the potential pitfalls associated with the use of cell lines obtained and processed casually require repeated emphasis. Numerous occasions where cell lines exchanged among co-operating laboratories have been contaminated with cells of other species have been detailed and documented elsewhere. For example, lines supposed to be human have been found to be monkey, mouse, or mongoose while others thought to be monkey or mink were identified as rat and dog. Similarly, the problem of intraspecies cross-contamination among cultured human cell lines has been recognized for over thirty years and detailed reviews are available on the subject. The loss of time and research funds as a result of these problems is incalculable. While bacterial and fungal contaminations represent an added concern, in most instances they are overt and easily detected, and are therefore of less serious consequence than the more insidious contaminations by mycoplasma. That the presence of these micro-organisms in cultured cell lines often negates research findings has been stated repeatedly. However, the prevalence of contaminated cultures in the research community suggests that the problem cannot be overemphasized.

2 Cell line banking

Cell lines pertinent for accessioning are selected by ATCC scientists and advisors during regular reviews of the literature. The originators themselves also frequently offer the lines directly for consideration. Detailed information with regard to specific groups of cell lines accessioned is available elsewhere. Generally, starter cultures or ampoules are obtained from the donor, and progeny are propagated according to instructions to yield the first 'token' freeze. Cultures derived from such token material are then subjected to critical characterizations. If these tests suggest that further efforts are warranted, the material is expanded to produce seed and distribution stocks. Note especially that the major authentication efforts are applied to cell populations in the initial seed stock of ampoules. The distribution stock consists of ampoules that are distributed on request to investigators. The reference seed stock, Conversely, is retained to generate further distribution stocks as the initial stock becomes depleted. Although this procedure has been developed to suit the needs of a large central repository, it is also applicable in smaller laboratories. Even where the number of cell lines and users may be limited, it is important to separate 'seed stock' from 'working or distribution stock'. Otherwise the frequent replacement of cultured material,

recommended to prevent phenotypic drift or senescence, may deplete valuable seed stock which may be difficult and expensive to replace. These various steps in the overall accessioning scheme are summarized in Figure 1. It is important to recognize that the characterized seed stock serves as a frozen 'reservoir' for production of distribution stocks over the years. Because seed stock ampoules are used to generate new distribution material, one can assure recipients that all the cultures obtained closely resemble those received ten or more years previously. This is a critical consideration for design of cell banking procedures. Problems associated with genetic instability, cell line selection, senescence, or transformation may be minimized or avoided entirely by strict adherence to this principle.

It is prudent to handle all primary tissues or cell lines not specifically shown to be free of adventitious agents as biohazards in a Class II vertical laminar flow hood (BS5726; NIH Spec. 03-112). This precaution protects both the cell culture technician and the laboratory from infection or contamination. Some recommend furthermore that all human tumour lines be treated with similar caution due to the known presence of oncogenes.
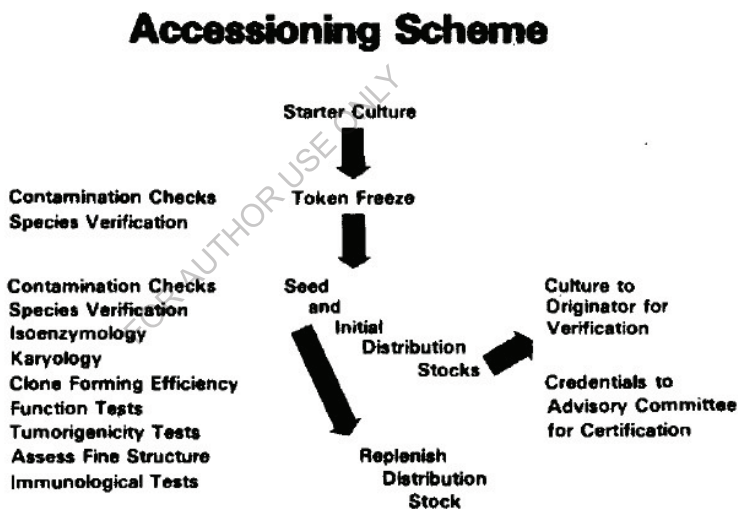
Figure 1. Accessioning scheme for new cell lines. Scheme illustrating a recommended plan for the accession of cell lines to be banked for general distribution. At least the first two of the various characterizations indicated on the left-hand side should be performed before release of any cell line.

3 Cell freezing and quantitation of recovery

Cellular damage induced by freezing and thawing is generally believed to be caused by intracellular ice crystals and osmotic effects. The addition of a cryoprotective agent, such as dimethyl sulfoxide (DMSO) or glycerol, and the selection of suitable freezing and thawing rates minimize cellular injury. While short-term storage of cell lines using mechanical freezers (-75°C) is possible, storage in liquid nitrogen (-196°C) or its vapour (to -135°C) is

much preferred. The use of liquid nitrogen refrigerators is advantageous not only because of the lower temperatures and, consequently, almost infinite storage times possible, but also due to the total absence of risk of mechanical failures and the prolonged holding times now available. Certainly for all but the smallest cell line banking activity, storage in a liquid nitrogen refrigerator is essential.

Two considerations on safety in processing cell lines require special emphasis. First, the cell culture technician may be endangered due to the possibility that liquid nitrogen can penetrate ampoules via hairline leaks during storage. On warming, rapid evaporation of the nitrogen within the confines of such an ampoule can cause a sharp explosion with shattered glass or plastic flying at high force almost instantaneously in all directions. Fortunately the frequency of such traumatic accidents declines dramatically as the operator gains experience in ampoule sealing and testing. However, even with highly accomplished laboratory workers, the remote possibility of explosion still exists. Thus, a protective face mask should be worn whenever ampoules are removed from liquid nitrogen storage and until they have been safely opened in the laminar flow hood. Secondly, DMSO can solubilize organic substances and, by virtue of its penetrability through rubber and skin, carry these to the circulation. Thus, special precautions should be exercised when using DMSO to avoid contamination with hazardous chemicals and minimize skin contact.

3.1 Equipment
3.1.1 Ampoules, marking, and sealing devices

A decision on whether to utilize glass or plastic ampoules will depend on the scale of operation and the extent of anticipated distribution. Glass ampoules can be sterilized, loaded with the appropriate cell suspension, and permanently sealed in comparatively large quantities. They are recommended for large lots of cells (20 ampoules or more) are prepared for long-term use or general distribution. Smaller numbers (1-20) of plastic ampoules are easier than glass to handle, mark, and seal. Problems with the seal may occur in some cases, however, especially if frequent handling or manipulations for shipment are necessary. The marking of ampoules requires special consideration in that legibility can easily be obscured as the ampoules are frozen, snapped on and off storage canes, transferred between freezers or shipping containers, and so forth. The use of paper labels, ballpoint pen, or standard laboratory markers all are problematical and this is especially true with glass ampoules. These should be labelled in advance with ceramic ink that can be heat annealed to the glass surface. The markings can be applied by hand with a straight pen or, if large lots are being processed, through use of a mechanical labeller (e.g., from Markem Co., Keene, New Hampshire).

Glass ampoules can best be sealed by pulling on the neck of the ampoule as it is rotated in the highest heat zone of the flame from a gas-oxygen torch. This pull-seal technique is preferred because it reduces the risk of permitting pin-hole leaks in the sealed tip. Torches for manual sealing can be obtained from scientific supply houses. For large lots, a torch can be attached to a semi-automatic sealing device available as Bench sealer model 161 from Morgan Sheet Metal Co., Sarasota, FL, USA.

3.1.2 Slow freezing apparatus

The optimum freezing rate for cell lines (for most cells about -1 °C/min) can be achieved through use of apparatus varying in complexity from a tailor-made styrofoam box to

a completely programmable freezing unit. The former should have a wall thickness of about 2 cm to approximate the -1 °C cooling rate when placed in a mechanical freezer at -70 °C. Alternatively, manufacturers of liquid nitrogen refrigerators supply adapted refrigerator neck plugs, at modest cost, which can be adjusted for slow freezing of numbers of ampoules. For those who produce larger quantities of ampoules and require more precise control of the freezing rate, a controlled rate freezer (e.g., Cryo-Med; Planer; Union Carbide) should be considered.

3.1.3 Liquid nitrogen refrigerators

Choice of an appropriate refrigerator will require considerations of economy, both in terms of liquid nitrogen consumption and initial outlay, storage capacity required, and desired ease of entry and retrieval. Freezers with narrower neck tube openings are generally more economical.

Ampoules of cells may be stored immersed in liquid nitrogen or in the vapour phase. The latter has the advantage that ampoules with pin-hole leaks will not be exposed to liquid so the danger of explosion *is* eliminated. The slightly higher temperature probably offers no disadvantage except perhaps with seed stocks retained for extremely long periods.

Useful accessories for refrigerators include roller bases for ease of movement, alarm systems to warn of dangerously low levels of liquid nitrogen, and racking systems, with larger refrigerators, for ready storage and recovery. Even when an automatic alarm system is used this should be backed-up with a regular manual check using a dip-stick. Electronic systems can fail and it has been known for both the automatic fill system and its back-up alarm to fail.

3.2 Preparation and freezing

Cultures in the late logarithmic or just pre-confluent phase of growth should be selected to give the highest possible initial viability. Treat the cultures with trypsin if necessary to produce a uniform single cell suspension as if for routine subcultivation and proceed.

3.3 Reconstitution and quantitating recovery

Rapid thawing of the cell suspension is essential for optimal recovery; a variety of methods may be used to quantitate cell recovery after freezing. Of course this is the first characterization step to be performed after preservation.

3.3.1 Dye exclusion for quantitating cell viability

A very approximate estimate of the viability of cells in a suspension may be obtained by the dye exclusion test. A solution or the dye in saline is added to the suspension and the percentage of cells which do not take up the stain is determined by direct count using a haemocrvtometer. Tiypan blue or erythrocin B are the stains most commonly used for this procedure. The former reportedly has a higher affinity for protein in solution than for non-viable cells. This may reduce the accuracy of the estimate if the suspension contains much more than 1 % serum. Furthermore, because solutions of erythrocin B are clear, microbial growth or precipitates are immediately apparent. This is not true for stock solutions of trypan blue.

3.3.2 Colony-forming efficiency

The dye exclusion test for cell viability generally overestimates recovery. For lines consisting of adherent cells, the colony-forming ability of cells from the reconstituted population represents a more accurate overall estimate of survival. Of course the choice of growth medium used, the substrate on which the colonies develop, and the incubation time all may have an effect on the end-result. Thus for comparisons among different freezes, conditions for selected lines must be standardized.

3.3.3 Proliferation in mass culture

The vitality of reconstituted cells from either adherent or non-adherent lines can also be documented by simply quantitating proliferation during the initial one to two weeks after recovery from liquid nitrogen.

4 Cell line authentications

In addition to recoverability from liquid nitrogen, the absolute minimum recommended authentication steps include verification of species and demonstration that the cell line is free of bacterial, fungal or mycoplasmal contamination.

4.1 Species verification

Species of origin can be determined for cell lines using isoenzyme profiles or by cytogenetics. These two methods are used at ATCC.

4.1.1 Isoenzyme profiles

Isozyme analyses performed on homogenates of cell lines from over 25 species have demonstrated the utility of this biochemical characteristic for species verification. By determining the mobilities of multiple isozyme systems one can identify the species of origin of cell lines with a high degree of certainty. The procedures are relatively straightforward, provide consistent results, and do not require expensive equipment. A kit (AuthentiKit™, Innovative Chemistry, Inc.) has been developed for this purpose. Pre-cast *1%* agarose gels on a polystyrene film backing, buffers, enzyme substrates with stabilizers, and appropriate control extracts are available. Specially constructed electrophoretic chambers are utilized after coupling to power supplies. Over 15 different enzyme systems can be evaluated. The advantages of the kit include the convenience of ready-made gels and reagents plus the significantly lower times required for electrophoretic separations (15-45 min). After drying, the gels can be retained to document cell line characteristics (Figure 2).

4.1.2 Cytogenetics

Karyologic techniques have been used to monitor for interspecies contamination among cell lines. In many instances the chromosomal constitutions are so dramatically different that even cursory microscopic observations are adequate. In others, as for example comparisons among cell lines from closely related primates, careful evaluation of banded preparations is required.

The standard method involves the swelling of metaphase-arrested cells by brief exposure to hypotonic saline (Table 1). The cells are then fixed, applied to slides to optimize

spreading, stained, and mounted for microscopic observation. The frequency of introduction of artefacts through this method will vary depending upon the cell line and the degree of experience. Rupturing of cells will occur, for example, and apparent losses or gains in chromosomes will result.

However, by counting the chromosomes in 50-100 well-spread mctaphases and recording the modal number the cytogeneticist can obtain a reliable estimate for a specific line.
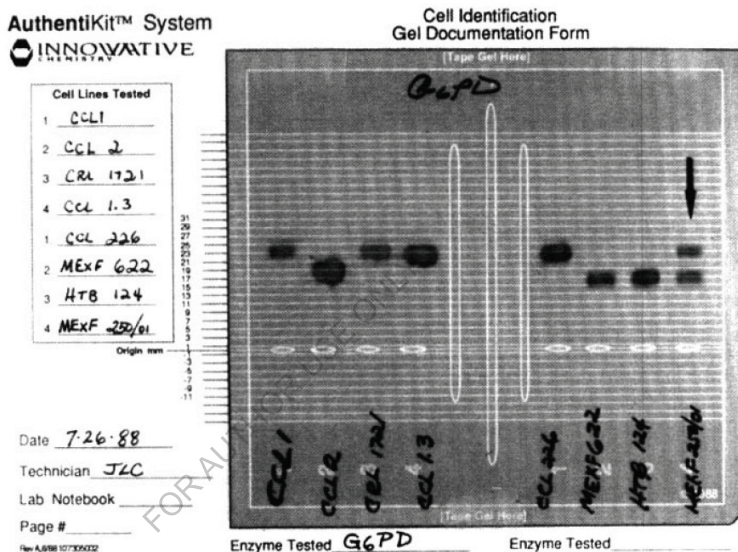
Figure 2. Isoenzyme get. An agarose gel processed for giucose-6-phosphate dehydrogenase using the AuthentiKit™. The gel was loaded with extracts from murine and human cells plus one murine/human mixture. Lanes *2* in the left section (LHS) and *2* and 3 in the right section (RHS) are human. Lanes 1, 3, and 4 (LHS) and 1 (RHS) are from extracts of murine lines. Lane 4 (RHS and arraiv) is from the mixed line.

Table 1. Stock solutions for routine karyology

Colcemid stock: 1 mg/50 ml double glass distilled water (DGDW); store frozen in small aliquots

KCl: 0.075 M in DGDW

Fixative: 3 parts anhydrous methyl alcohol, 1 part glacial acetic acid (combine just before use)

Acetic orcein stain: 2 g natural orcein dissolved in 100 ml of 45% acetic acid

Giemsa stain: 10% in 0.01 M phosphate buffer at pH 7 (solution available commercially)

The karyotype is constructed by cutting chromosomes from a photomicrograph either manually or using a software package and arranging them according to arm length, position of centromere, presence of secondary constrictions, and so forth. Consult the Atlas of mammalian chromosomes for examples of conventionally stained preparations from over 550 species. For a more critical karyotypic analysis, chromosome banding techniques are required.

4.2 Tests for microbial contamination

The tests included here are suitable for detection of most micro-organisms that would be expected to survive as contaminants in cell lines or culture fluids. Techniques for detecting protozoan contamination are not presented as these organisms are rarely found in continuous lines and descriptions of the methodology are available elsewhere. Commercial dry powders are entirely satisfactory for preparation of test media for bacteria, fungi, and mycoplasma provided that positive controls are included at least with the initial trials on each lot obtained.

4.2.1 Detection of bacteria and fungi

To examine cell cultures or suspect media for bacterial or fungal contaminants, Microscopic examination is only sufficient for detection of gross contaminations and even some of these cannot be readily detected by simple observation. Therefore, an extensive series of culture tests is also required to provide reasonable assurance that a cell line stock or medium is free of fungi and bacteria.

4.2.2 Mycoplasma detection

Contamination of cell cultures by mycoplasma can be a much more insidious problem than that created by growth of bacteria or fungi. While the presence of some mycoplasma species may be apparent due to the degenerative effects induced, others metabolize and proliferate actively in the culture without producing any overt morphological change in the contaminated cell line. Thus cell culture studies relating to metabolism, surface receptors, virus-host interactions, and so forth are certainly suspect, if not negated in interpretation entirely, when conducted with cell lines harbouring mycoplasma. Nine general methods are available for detection of mycoplasma contamination. The direct culture test, a sensitive PCR analytical kit, and the 'indirect' test employing a bisbenzimidazole fluorochrome stain (Hoechst 33258) for DNA are used routinely at the ATCC to check incoming cell lines and all tell distribution stocks for mycoplasma. The serum, yeast extract, and other ingredients used for mycoplasma isolation and propagation should be pre-tested for absence of toxicity and for growthpromoting properties before use. Positive controls consisting of *M.arginim. M.orale* and A. *laidlawii* are recommended because they are among the most prominent species isolated from cultured cells.
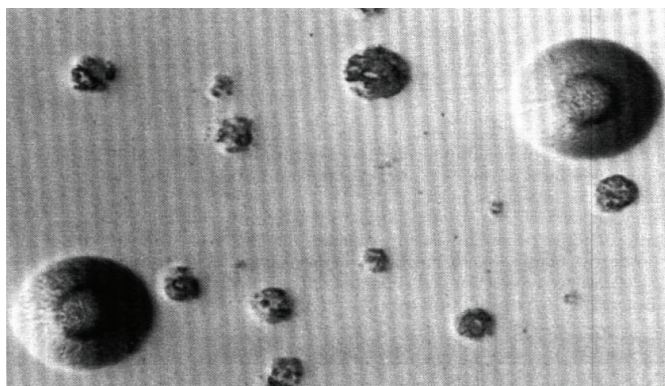
Figure 3. Mycoplasma colonies. Micro-photograph showing colonies of mycoplasma in agar plate. The smaller material between colonies is from the inoculum of cultured cells and debris.

Table 2. Suggested regimen for detecting bacterial or fungal contamination in cell cultures

Test medium	Temperature (°C)	Aerobic state	Observation time (days)
Blood agar with fresh defibrinated rabbit blood (5%)	37	Aerobic	14
	37	Anaerobic	14
Thioglycollate broth	37	Aerobic	14
	26		
Trypticase soy broth	37	Aerobic	14
	26		
Brain heart infusion broth	37	Aerobic	14
	26		
Sabouraud broth	37	Aerobic	21
	26		
YM broth	37	Aerobic	21
	26		
Nutrient broth with 2% yeast extract	37	Aerobic	21
	26		

As many strains of mycoplasma, especially *M. hyorhinis,* are difficult or impossible to cultivate in artificial media, an 'indirect' test method should also be included. Positive and negative controls should be included with each test series. For this indirect test the Vero or 3T6 cells inoculated with medium only serve as negative controls. *M. hyorhinis, M, argmini, M. orale,* and *A. laidlawti* are suitable controls with the order of preference as listed. The nucleic acid of the organisms is visible as particulate or fibrillar matter over the cytoplasm with the cultured cell nuclei more prominent (Figure 4). With heavy infections, intercellular spaces also show staining.
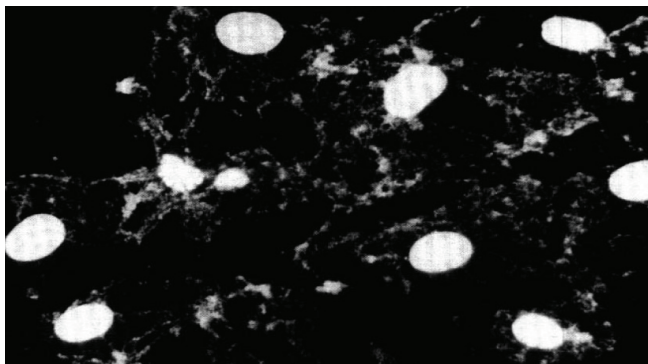
Figure 4. Hoechst staining of mycoplasma. Microphotograph showing mycoplasmal DNA demonstrated by Hoechst staining. The larger fluorescing bodies are nuclei of the substrate cell line Vero.

Preparation of positive controls, i.e., deliberately infecting cultures, creates a potential hazard to other stocks, so infected cultures should be prepared at a time when, or in a place where, other cultures will not be at risk. Fix and store positive controls at -4°C over desiccant until required. In all quality control work with mycoplasma or unknown cultures it is prudent to work in a vertical laminar flow hood, preferably isolated from other standard cell culture activity. One should be aware continually of the danger of contaminating clean cultures by aerosols from mycoplasma-containing cultures manipulated in the same area or manually during processing of multiple culture flasks. Appropriate disinfection between uses of a hood or work area is strongly recommended.

Various polymerase chain reaction (PCR) tests for mycoplasma have been developed, including those used at ATCC. Eight commonly encountered mycoplasma contaminants can be detected: *M. arginini, M. fermcntans, M. homims, M.hyorhims, M. orak, M, pirum, M. sdivarium,* and *Acholeplasma laidlawii;* which account for more than 95% of mycoplasma contaminants in cell cultures. Mycoplasma DNA in plants and insects can also be detected. The amplification product can be used to identify the species of mycoplasma by size determination and restriction enzyme digestion.

4.2.3 Testing for the presence of viruses

Of the various tests applied for detection of adventitious agents associated with cultured cells, those for endogenous and contaminant viruses are the most problematical. Development of an overt and characteristic cytopathogenic effect (CPE) will certainly provide an early indication of viral contamination. The absence of CPE, however, definitely does not indicate that the culture is vims-free. In fact, persistent or latent infections may exist in cell lines and remain undetected until the appropriate biological, immunological, cytological, ultra structural, and/or biochemical tests are applied. Additional host systems or manipulations, for example, treatment with halogenated nucleosides, may be required for virus activation and isolation. Molecular techniques for detection and identification of

specific viral infections may be diagnostic. In this regard, a non-radioactive method has been adapted at ATCC to screen for human immunodeficiency (HIV) and hepatitis viruses (e.g., HBV) in human fetal cells and tissues. As cited by David Kellogg and co-workers SK38 and SK39 primers have been successfully used to detect HIV-I in infants born to seropositive mothers.

Table 3. Reagents for detection of HIV

Primers:	SK385'-TTTGGTCCGCCTATCCCAGTAGGAGAAAT-3'
	SK39 5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'
DNA probes:	SK-19 ATCCTGGGATTAAATAAMTAGTAAGAATGTATAGCCCTAC
Positive control:	ATCC cell line CRL-8993 (8E5)
Negative control:	ATCC cell line CCI-171 (MRC-5

Table 4. Reagents for detection of HBV

Primers:	MD06 (5')hCTTGGATCCTATGGGAGTGG
	MD03 (5')-CTCAAGCTTCATCATCCATATA
DNA probes:	MD09 (5') GCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTC
Positive control:	ATCC cell line HB-8064 (Hep-SB)
Negative control:	ATCC cell line CCL-2

Table 5. Solutions for HIV/HBV assays

1 x TBE buffer:	0.05 M Tris, 0.05 M boric acid, 1 mM EDTA pH 8.0
20 x SSC buffer:	3 M NaCI, 0.3 M Na3 citrate'2H20 pH 7.0
Lysis buffer:	0.15 M NaCI, 0.1 M EDTA, 0.02 M Tris, 1% (w/v) SDS pH 8.0
	20 mg/ml proteinase K stock in 0.15 M NaCI
	10 mg/ml RNase stock in 10 mM Tris pH 7.5
Maleic acid:	0.1 M maleic acid, 0.15 M NaCI pH 7.5
Standard hybridization	0.1% W lauroylsarcosine, 0.02% SDS, 1% blocking reagent,
buffer (1 litre):	125 ml of 20 x SSC
Washing buffer:	0.1 M maleic acid, 0.15 M NaCI, 0.3% (w/v) Tween 20 pH 7.5

These oligonucleotides primers (SK38, SK39) amplify a 115 bp fragment of the HIV gag gene (Table 3). MD06 and MD03 oligonucleotides are unique for HBV. The first oligonucleotide (MD06) spans position map genome 636-648, and the second position (MD03) 736-746. During the PCR amplification those two nucleotides amplify the synthesis of the 110 bp fragments (Table 4). The amplicons are separated by a 10% polyacrylamide gel, then transferred to a nylon membrane by electrophoretic transblotting, and detected by hybridization with digoxigenin labelled oligonucleotides and chemiluminescence (Tables). Equipment, reagents, and supplies for these methods are commercially available. HBV amplicons and controls revealed by chemiluminesce are provided as Figure 5.

It should be emphasized that, in spite of these screens, latent viruses and viruses which do not produce overt CPE or haemadsorption will escape detection. Some of these could be potentially dangerous for the cell culture technician. For additional detail and methodology consult refs 20-23.

Base Pair

Figure 5, Photo of PCR for HBV. Screen for human hepatitis B sequences in fetal cells. Positive controls show amplicons from cell lines Hep-3B and PLC/PRF5 known to be infected with hepatitis B (ATCC HB-8064 and CRL-8024). Negative control artel test cultures are indicated by letters WHC.

4.3 Testing for intraspecies cross-contamination

With the dramatic increase in numbers of cell lines being developed, the risk of intraspecies cross-contamination rises proportionately. The problem is especially acute in laboratories where work is in progress with the many different cell lines of human and murine origin (hybridomas) available today. Tests for unique karyology, polymorphic isoenzymes and DNA, and surface marker antigens are all important tools to detect cellular cross-contamination within a given species.

4.3.1 Giemsa banding

A powerful method for cell identification is karyotype analysis after treatment with trypsin and the Giemsa stain (Giemsa or G-banding). The banding patterns are characteristic for each chromosome pair and permit recognition by an experienced cytogeneticist even of comparatively minor inversions, deletions, or translocations. Many lines retain multiple marker chromosomes, readily recognizable by this method, which serve to identify the cells specifically and positively.

Figure 6. Giemsa banded chromosomes from the cell line ATCC.CCL-208 (4MBr-5) isolated from a rhesus monkey bronchus.

Completely dried slides are used to examine under bright-field, oil-immersion planapochromat objectives without coverslip. Oil can be placed directly on the slide. However, care must be taken not to scratch the cell surface. Oil must be removed as completely as possible immediately after the use of slides. Generally a few changes in xylene should be satisfactory. A typical banded preparation is shown as Figure 6.

4.3.2 DNA fingerprinting

DNA fingerprinting of human cell lines provides the resolution necessary to confirm the authenticity of a cell line and to rule out the possibility of cell line cross-contamination. DNA fingerprinting became possible with identification of hypervariable repetitive minisatellite (base repetitive motif is 10-20 bp) DNA. Traditional use of these loci involved the laborious, time-consuming procedure of Southern blotting. Two strategies have been used when working with VNTR loci. The first strategy employs a multilocus probe that can simultaneously illuminate the genotype at many loci. Although highly informative, this strategy is problematic as a routine screening technique due to the difficulty in interpreting the complex multiple banding patterns. The alternative strategy is to use a cocktail of less informative single locus VNTR probes. When the single locus probe genotypes are combined, a level of discrimination similar to multilocus VNTR fingerprints is possible with the simplicity of interpreting two band patterns. The adaptation of the polymerase chain reaction (PCR) has removed the need to use Southern blotting when examining these VNTR loci which means that loci can be typed in hours rather than days.

Smaller microsatellite (base repetitive motif is 2-6 bp) loci have been identified as well, Short tandem repeat (STR) loci in differentiating humans at the DNA level. One significant advantage enjoyed by STR loci over their minisatellite cousins is their small size. The small size of STR loci allows multiplex PCR reactions to be developed in which many loci are simultaneously examined in a single reaction.When authenticating a new line, it is recommended that DNA be extracted from the cell line using a traditional liquid extraction method as this tends to minimize STR artefacts which may complicate allele assignment. For validation of subsequent passages of the cell line, more expedient DNA techniques may be used, which may produce ambiguous allele assignments. Generally, comparison with the authentic DNA fingerprint easily resolves these ambiguities. Although the STR system

presented utilizes fluorescent labels and an automated collection device, it should be noted that various SIR formats have been developed that use isotopic, chemiluminescent, or silver staining to detect and type these loci with similar accuracy. One facet of DNA fingerprinting data, which cannot be overemphasized, is the need to convert PCR fragment sizes into alleles when developing genotypes for cell lines. This requires the development of an allelic ladder which contains all of the commonly observed alleles for each STR locus under examination. This ladder must be run on every gel, as it will serve to normalize the data and minimize the errors that inevitably crop up when comparing fragment size data between different gels. More importantly, this allows different laboratories, which may use different STR formats, to compare their results in *a* straightforward, unambiguous manner. Typical STR profiling results are presented in the electropherograms (Figures 7 and 8). Figure 7 represents the data generated when analysing two cell lines derived from the same patient. Figure 8 compares the STR profiles generated from two unrelated cell lines.
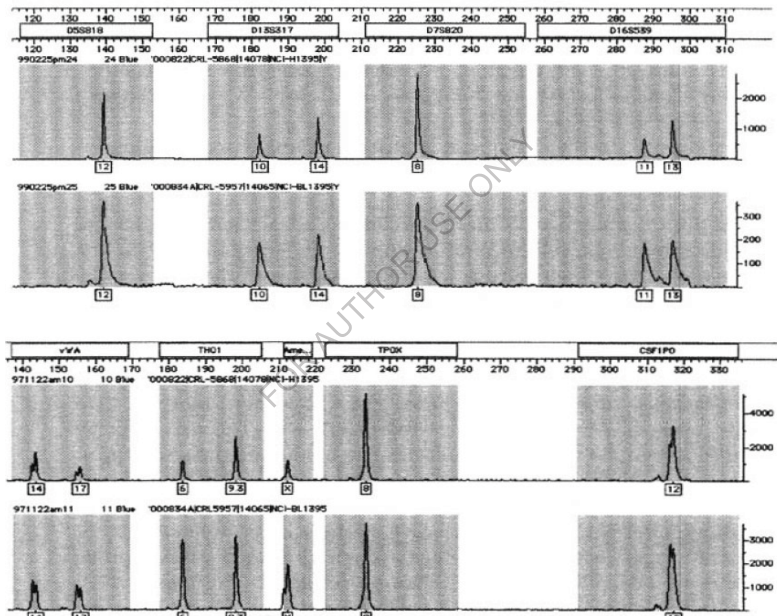
Figure 7. DNA profiles of cell lines from the same patient. Comparison of the identical STR profiles for the EBV-transformed lymphoblast line CRL-5957 and the tumour line CRL-5868 derived from the same female patient. Upper tracings represent the four STR loci D5SS18, D13S317, D7S820, and D16S539. The lower tracings represent the four STR loci vWA, TH01, TPOX, and CSF1PO as well as the amelogenin locus used for gender identification.
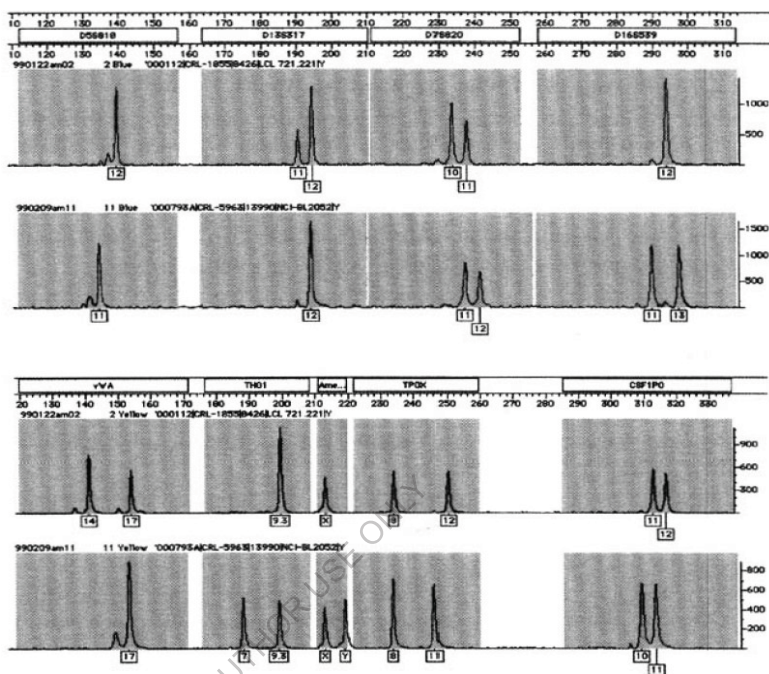
Figure 8. DNA profiles of two unrelated cell lines. Comparison of unique STR profiles for the unrelated male cell line CRL-5963 and female cell line CRL-1855, Upper tracings represent the four STR loci D5S818, D13S317, D7S820, and D16S539. The lower tracings represent the four STR loci vWA, TH01, TPOX, and CSF1PO as well as the amelogenin locus used for gender identification.

Table 6. Laboratory stock buffers and reagents

1 x PBS:	135 mM NaCI, 2.5 mM KCI, 10 mM Na$_2$HP0$_4$, 2 mM KH$_2$PO$_4$ pH 7.4
20% SDS	
6 M NaCI	
Isopropanol	
70% ethanol	
Low TE buffer:	10 mM Tris, 0.1 mM EDTA pH 7.6

Table 7. Vendor reagents and supplies

Cell lysis solution (Gentra Systems, Cat. No. D-50K2)
RNase A solution (Gentra Systems, Cat. No. D-50K6)
FTA classic format card (Life Technologies, Cat. No. 10786010) includes:
FTA purification reagent
TE-a (low TE buffer, supplied with kit)
UltraBARRIER pouch (Life Technologies, Cat. No. 64030026)
HARRIS micro-punch (Life Technologies. Cat. No. 10786069)
GenePrint™ PowerPlex1* 1.2 System (Promega, Cat. No. DC6101) includes:
Gold STAR buffer
PowerPlex 10 X primer pair mix
AmpliTaq Gold™ (Perkin Elmer, Cat. No. N808-0241)
Mineral oil (Perkin Elmer, Cat. No, 0186-2302)
GS-500 ROX (PE/ABD, Cat. No. 401734)
Formamide (Sigma, Cat. No. F-9037)
6% polyacrylamide (8.3 M urea) Burst-Pak™ Gel (Owl Scientific, Cat, No, SEQ-6-10)
5 x TBE liquid concentrate (Amresco, Cat. No. J885-1L)

4.4 Characterizations and cell line availability

A host of additional cell line characterizations may be considered depending upon the tissue type of origin and the intended use for given cell lines. For example, analysis of fine-structure, immunological tests for cytoskeletal and tissue-specific proteins, or assays for monoclonal antibody secreted may be required. Authenticated cell lines may be obtained from national banks established over the past 10-30 years. Such public repositories include the ATCC, the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research in Camden, NJ, USA, the German Collection of Microorganisms and Cell Cultures (DSMZ) in Braunschweig, the European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK, the Japanese Cell Bank for Cancer Research at the National Institute of Hygiene Services in Tokyo, and the Riken Cell Bank in Tsukuba City, Japan.

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Chapter 5
Serum-free media

1 Introduction

The removal of serum or other undefined components from cell culture medium involves a multifaceted approach. This is mainly due to the interactive nature of cell culture systems. Cell culture systems require various components: basal nutrients, a set of supplements that convey information, such as growth factors, mitogens, hormones, and cytokines and for anchorage-dependent cells, a substrate. In designing any cell culture system attention must be paid to all these components and their interactions. The role of the basal nutrient component needs to be addressed in a systematic manner. For normal eukaryotic cells, the optimization of the basal nutrients is an important aspect in the generation of serum-free media. The removal of an essential nutrient, such as an amino acid, vitamin, or mineral would effectively inhibit the activity of the cells in a serum-free culture environment. It has been demonstrated qualitatively that various cell types require the same nutrients but that quantitatively there are significant differences. It is reasonable to assume that there is an optimized concentration for a set of nutrients for a particular function for a particular cell type.

2 Role of serum and other undefined tissue extracts in cell culture systems

In media these undefined supplements (serum, pituitary brain, and other extracts) provide not only essential nutrients and attachment factors but also act as detoxificants. The undefined nature of these supplements and their potential for carrying adventitious agents and determining the phenotype of cells motivates their avoidance or minimization whenever possible. These supplements can contain inhibitors or promoters of the processes under study. In the bioprocessing industry the contribution of these supplements to the final product necessitates extensive downstream purification. In addition, the cell phenotype can be better controlled in the absence of serum.

3 Response curves
3.1 Proliferating cultures

The quantitative evaluation of nutrient requirements necessitates titration of the basal components in a systematic manner. The responses evaluated can be any cellular characteristic: proliferation, product secretion, or expression of a differentiated function. Two types of responses are possible: the essential nutrient response, and the non-essential nutrient response (Figure 1). The essential response is characterized by a zero response to zero concentration, followed by a positive increase to rising concentrations, a plateau region where no increase in response is observed with higher concentration, and finally an inhibitory region. The slope of the positive response, the length of the plateau, and the slope of the inhibitory regions are dependent on the nutrient and cell under study. The aim of this exercise is to determine the concentration of the nutrient that is represented by the middle of the plateau.

In optimizing the concentration of a nutrient, amino acids represent the largest group where interactions occur, because there are common transport systems for amino acids. These systems interact in a positive or negative manner. A consequence of this is that by changing

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the concentration of one amino acid it is possible to influence the response curve of another amino acid. By placing the concentration of a component in the middle of the plateau region, a shift in any direction will minimize the risk of entering a deficient or inhibitory area. The non-essential condition is characterized by a significant response at zero concentration of the nutrient. There may also be a response to increasing concentrations of the nutrient. If the desired outcome is maximal proliferation, then the concentration of the non-essential nutrient also needs to be optimized.

Examples of this are observed in Figures 2 and 3. In Figure 2, L-phenylalanine and L-tyrosine are titrated using normal human aortic smooth muscle cells (AOSMC). The L-tyrosine titration demonstrates that in the absence of L-tyrosine, proliferation close to maximum is observed. However, in Figure 3, where Lphenylalanine and L-tyrosine are titrated using normal human bronchial/tracheal epithelial cells (NHBE), L-tyrosine appears to be an essential nutrient. In whole animals L-phenylalanine can be converted to L-tyrosine by L-phenylalanine hydroxylase. This metabolism has been observed in various other cell culture systems but not with NHBE. In a similar fashion, titrating the amino acid pair L-methionine and L-cysteine (Figures 4 and 5), it is seen that Lcysteine is not an essential amino acid for AOSMC, whereas in NHBE, L-cysteine is essential. L-cysteine is synthesized from L-serine and L-methionine in the liver and brain and in fibroblasts expressing cystathionine synthetase.

Nutrient requirements for cells in culture are determined best in clonal growth assays. At high density, cells are able to condition the medium by excreting essential factors. In *a* clonal growth assay the volume of medium is too large for the small number of cells to effectively condition the medium. Consequently, the cells are dependent on the nutrients present in the medium and a deficiency may be observed.
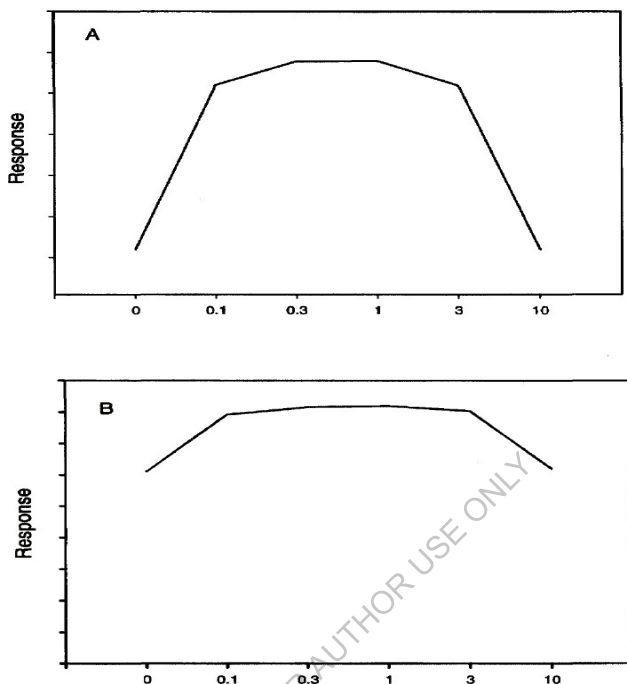
Figure 1. Idealized response of an essential (A) and non-essential (B) nutrient. Based on these responses it is possible to determine the optimized concentration of the nutrient. Note that in the absence of an essential nutrient (one that cannot be synthesized by the cell) no response is observed at zero concentration. On addition of the essential nutrient a dose response is observed: an increase in concentration results in an increase in response. At higher concentrations a plateau is observed where there is no increase in response with increased concentration. The third part of the response is characterized by a decrease in response with increasing concentration of nutrient. In the case of the non-essential nutrient a significant response is observed at zero concentration. However, further addition of the non-essential nutrient may result in an enhanced response. Although the non-essential nutrient can be synthesized by the cell it may not be synthesized at rates sufficient to meet the maximum requirement and/or the addition of the nutrient may relieve the metabolic load required for its synthesis.

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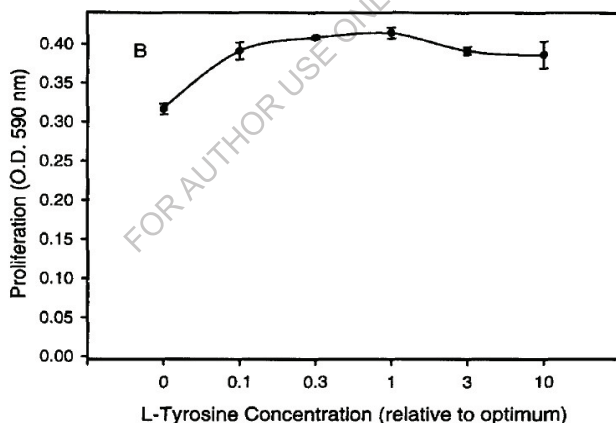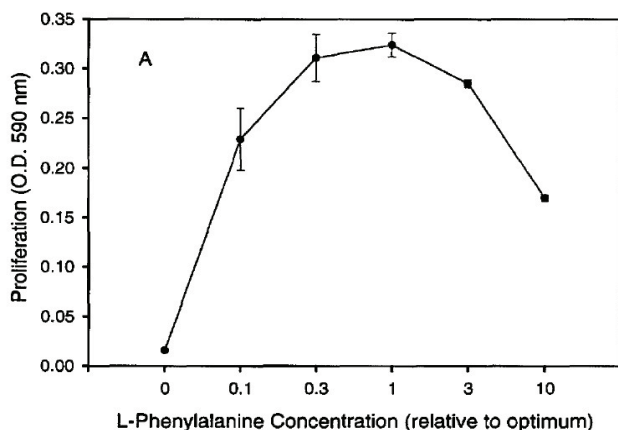
Figure 2. Titration of L-phenylalanine (A) and L-tyrosine (B) using AOSMC. L-phenylalanine is demonstrating essential nutrient behaviour while L-tyrosine is demonstrating non-essential nutrient behaviour. In the absence of L-tyrosine, L-phenylalanine may be contributing to the L-tyrosine pool through the action of phenylalanine hydroxylase, but there may also be protein breakdown contributing to the L-tyrosine pool. The cells were plated at 200 cells/cm^2 and kept in culture for 10-11 days without a medium change and then fixed in formaldehyde and stained with crystal violet. Quantitation was performed by extracting the crystal violet and reading the optical density at 590 nm. The optical density of extracted crystal violet is correlated with cell number during logarithmic growth. No serum or other tissue extract was present in the medium unless otherwise stated. The values on the *x* axis refer to multiples of the log of the optimal concentration.
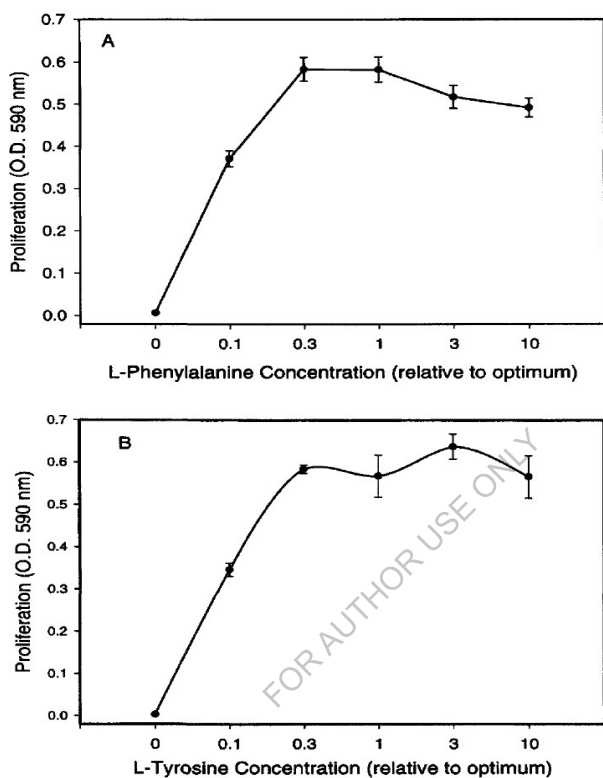
Figure 3. Titration of L-phenylalanine (A) and L-tyrosine (B) in NHBE. Both L-phenylalanine and L-tyrosine behave as essential nutrients. The values on the x axis refer to multiples of the log of the optimal concentration. These cells were plated at 100 cells/cm^2 and kept in culture for seven days without a medium change. No serum or other tissue extract was present in the medium unless otherwise stated. The values on the *x* axis refer to multiples of the log of the optimal concentration. Quantitation was performed as described in Figure 2.
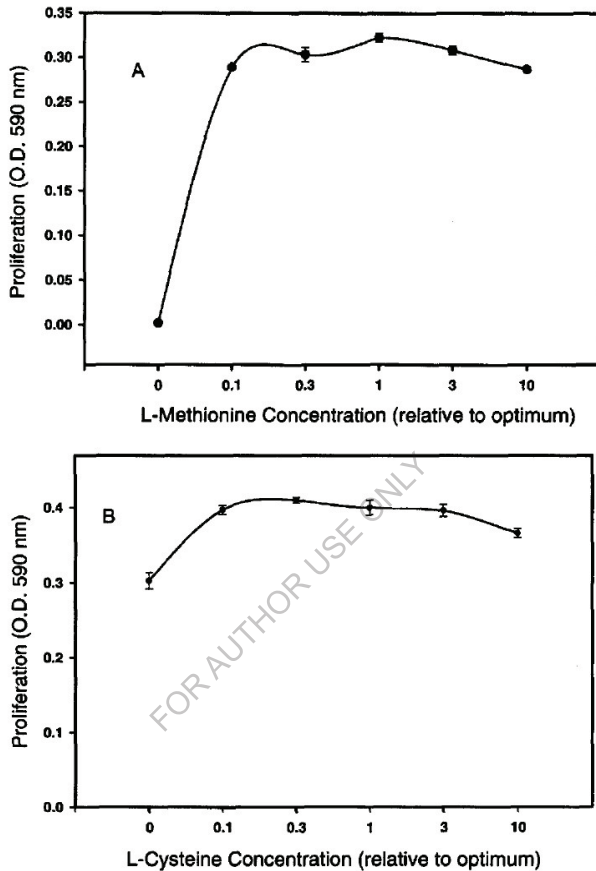
Figure 4. Titration of L-methionine (A) and L-cysteine (B) in AOSMC. L-methionine is demonstrating essential nutrient behaviour while L-cysteine is demonstrating non-essential nutrient behaviour. L-cysteine can be synthesized from L-serine which provides the carbon chain and L-methionine which provides the sulfur atom. No serum or other tissue extract was present in the medium. The values on the *x* axis refer to multiples of the log of the optimal concentration. Conditions are similar to those stated in Figure 2.

One basal component with profound effects on cell proliferation is sodium chloride (NaCl). Figure 6 demonstrates the response of normal human epidermal keratinocytes (NHEK) to various concentrations of NaCl. The response is characterized by a relatively sharp peak. The sensitivity to NaCl may be due to the energy expenditure associated with the maintenance of the Na^+-K^+-ATPase membrane transport.
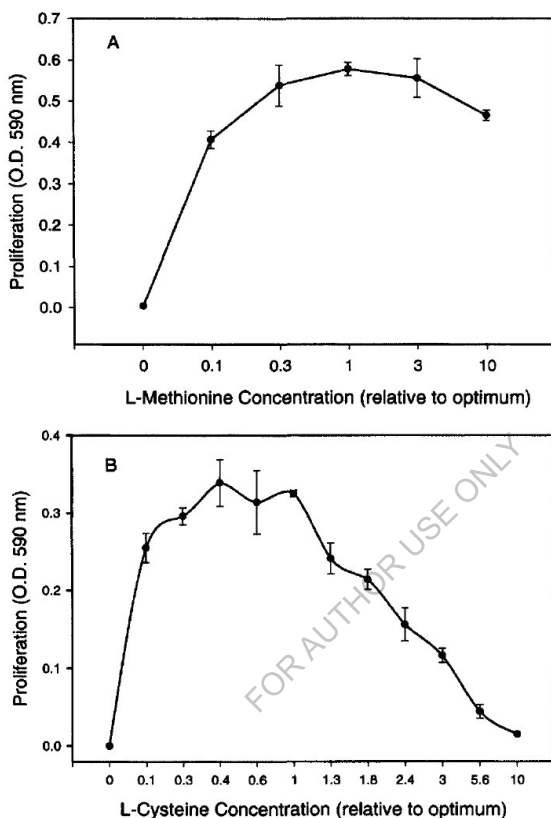
Figure 5. Titration of L-methionine (A) and L-cysteine (B) in NHBE. Both L-methionine and L-cysteine behave as essential nutrients. The values on the x axis refer to multiples of the log of the optimal concentration. These cells were plated at 100 cells/cm^2 and kept in culture for seven days without a medium change. No serum or other tissue extract was present in the medium unless otherwise stated. The values on the *x* axis refer to multiples of the log of the optimal concentration. Quantitation was performed as described in Figure 2.

3.2 Non-proliferating cultures: Primary Cells (ex: hepatocytes)

Normal adult rhesus monkey hepatocytes do not proliferate to any significant extent. A characteristic of hepatocytes is cytochrome P450 activity and the medium optimization uses this activity as an end-point. In Figure 7 the titration of two amino acids is depicted using hepatocytes exposed for 48 hours to 3-methylcholanthrene. A homologous series of the n-alkyl ethers of phenoxazone (alkoxyresorufins) is available for the study of various

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cytochrome P450s. The phenoxazone ethers are hydroxylated and 0-dealkylated to a common fluorescent metabolite, resorufin, which fluoresces at 590 nm when excited at 530 nm. The particular phenoxazone ether used to measure cytochrome P450 upon 3-methylcholanthrene induction was ethoxyphenoxazone. The L-leucine titration demonstrates a dose response, but no dose response is observed for L-tyrosine. L-leucine is demonstrating essential amino acid activity while L-tyrosine is not, and this may be due to the conversion of L-phenylalanine to L-tyrosine by phenylalanine hydroxylase.
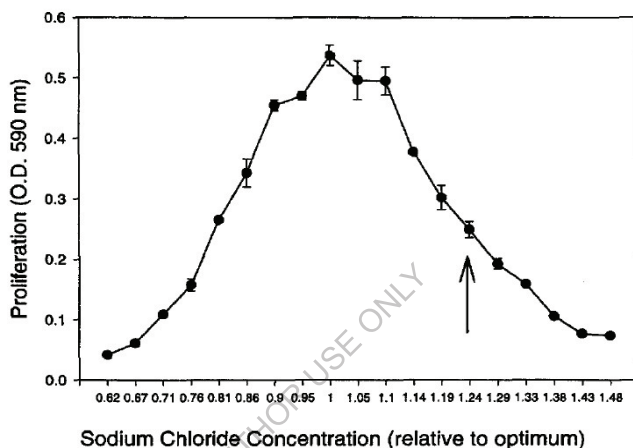
Figure 6. Response of normal human keratinocytes to various concentrations of NaCl. Sodium chloride demonstrates essential nutrient behaviour, which is not surprising given the various functions NaCl executes. Of the many functions of sodium chloride, influence over the osmolarity of the medium is one of the most profound. As can be seen from the graph, a 15-20% change in concentration has significant effects. The arrow refers to the concentration of NaCl before the optimization process. No serum or other tissue extract was present in the medium. The values on the *x* axis refer to multiples of the log of the optimal concentration. Quantitation was performed as described in Figure 2.

4 Antimicrobials, phenol red, Hepes, and light

Cell culture systems have a high growth potential for micro-organisms. During primary culture, control of the microbial load is highly desirable. However, once the culture is established, antimicrobials are unnecessary in small scale cultures and potentially could mask occult infection. In attempting to establish defined systems the use of antimicrobials and phenol red is debatable; however, in the bioprocessing industry where thousands of litres are at stake a microbial contamination would be a financial disaster.
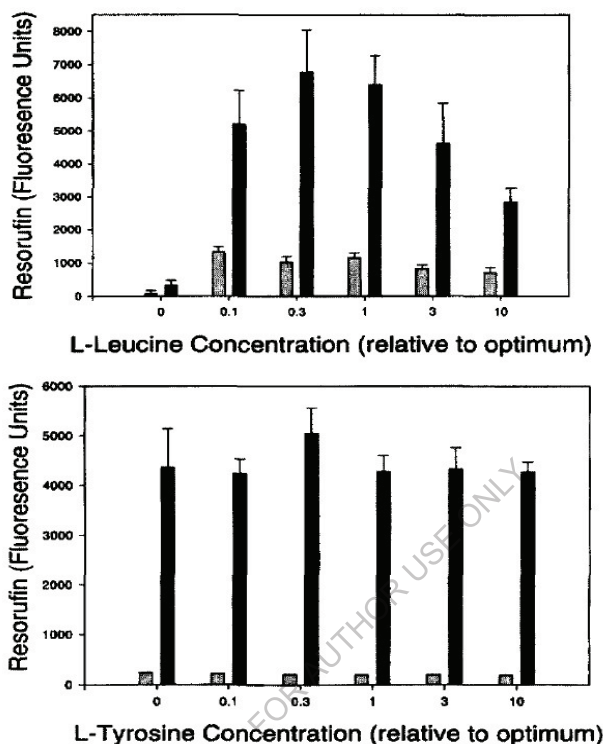
Figure 7. Response of Rhesus monkey hepatocytes to varying concentrations of L-leucine and L-tyrosine. The isolated hepatocytes were inoculated at 105 cells/cm^2 on collagen coated plates. After eight days in culture the hepatocytes were stimulated with 2 μM 3-methycholanthrene for an additional two days. 3-Methylcholanthrene is known to increase the expression of cytochrome P450 1A1/2. At that time 7-ethoxyphenoxazone was added for 40 min. After the incubation period the fluorescence of the 7-hydroxyphenoxazone, also known as resorufin (the product of the reaction), was read in the CytoFluor reader set at excitation 530 nm and emission of 590 nm. Each concentration has two bars: the smaller bar represents the fluorescence of the unstimulated hepatocytes and the larger bar represents the fluorescence of the stimulated hepatocytes. L-leucine demonstrates essential nutrient behaviour and L-tyrosine non-essential nutrient behaviour. The liver contains phenylalanine hydroxylase producing L-tyrosine and this is the most likely interpretation of the non-essential behaviour of L-tyrosine in this experiment.

4.1 Phenol red

Phenol red is widely used as a pH indicator in cell culture media. Unfortunately, phenol red or contaminants can interact unfavourably with cultured cells, causing toxicity and oestrogenic activity. Sodium-potassium ion homeostasis is perturbed in serum-free medium containing phenol red and the effect is neutralized by serum or serum albumin. A contaminant that became toxic when the pH rose above 7.4 but was virtually inert at lower pH values. HPLC analysis revealed many contaminating peaks and identified the cytotoxicity with a particular peak that differed from the oestrogenic activity. Anti-oestrogens frequently suppress growth below that of control cells not treated with oestrogen, even after careful steps have been taken to eliminate all oestrogen from the serum. The oestrogenic activity is due to a lipophilic impurity that binds to the oestrogen receptor with an affinity 50% that of oestradiol. Figure 8 demonstrates the response of four different strains of normal human bronchial/tracheal epithelial cells (NHBE) to varying concentrations of phenol red. These cells were cultured in the absence of bovine pituitary extract (BPE) and all experienced inhibition of proliferation. In the presence of BPE, at 3.3 x 10^{-6} M phenol red, the cells demonstrated an apparent neutralization of the phenol red inhibitory effect.
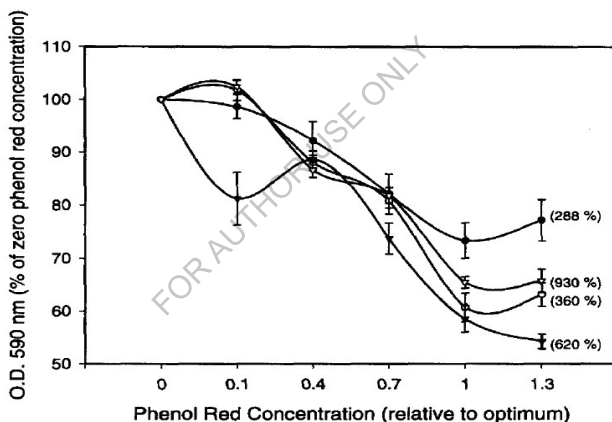
Figure 8. Responses of four different strains of NHBE to various concentrations of one lot of phenol red. These cells were inoculated at 10^{3} cells/cm^{2} and kept in culture for five to seven days. The cells were cultured in the absence of any undefined components. In each strain phenol red demonstrated a toxic effect at the recommended concentration, 1 on the x axis (3.3 x 10^{-6} M). The addition of bovine pituitary extract (BPE) at 3.3 x 10^{-6} M phenol red resulted in an increase in proliferation. This value is depicted in brackets as a per cent of the value in the absence of BPE. Quantitation was performed as described in Figure 2.

4.2 Gentamicin

Gentamicin belongs to the aminoglycoside family of antibiotics. These antibiotics are nephrotoxins *in vivo* and *in vitro*. However, toxicity is not exclusively localized to the kidney: the ear, heart, and the cornea have all demonstrated susceptibility.
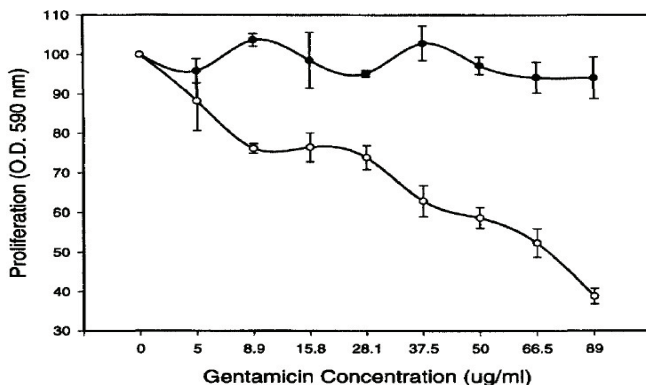
Figure 9. The response of NHBE to various concentrations of gentamicin from two different lots. These cells were inoculated at 100 cells/cm^2 and left in culture for seven days without a medium change. There were no undefined components in the medium. One lot of gentamicin demonstrated toxicity while the other did not. Each lot of gentamicin needs to be evaluated for toxicity. Quantitation was performed as described in Figure 2.

The mechanisms of toxicity range from free radical formation to calcium transport inhibition. Hepatocyte metabolic conversion of gentamicin to a compound toxic to sensory cells from the inner ear is another mechanism of toxicity. Gentamicin is toxic *in vitro* to corneal epithelial cells, fibroblasts and hepatocytes. The influence of two different lots of gentamicin on the proliferation of NHBE is shown in Figure 9, indicating that one lot was inhibitory.

4.3 HEPES

HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) is a zwitterionic organic chemical buffering agent; one of the twelve Good's buffers. HEPES is widely used in cell culture, largely because it is better at maintaining physiological pH despite changes in carbon dioxide concentration (produced by cellular respiration) when compared to bicarbonate buffers, which are also commonly used in cell culture. The dissociation of water decreases with falling temperature, but the dissociation constants (pK) of many other buffers do not change much with temperature. HEPES is like water in that its dissociation decreases as the temperature decreases. This makes HEPES a more effective buffering agent for maintaining enzyme structure and function at low temperatures.

HEPES has been used extensively in cell culture due to its excellent buffering capacity in the physiological range and low binding of cations but it can be toxic. For example, cytoplasmic vacuoles developed in chick embryo epiphyseal chondrocytes and membrane inclusion bodies in human dermal fibroblasts that resolved when HEPES was replaced by another buffer.

The toxicity exhibited by HEPES appears to be mediated by the production of reactive oxygen species. The interaction of HEPES-buffered culture medium with fluorescent light at

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room temperature resulted in toxicity to V79 Chinese hamster cells. The toxicity was prevented by shielding or the inclusion of catalase in the medium, implicating extracellular hydrogen peroxide as the toxic agent. In endothelial cells, HEPES stimulates the production of toxic oxygen metabolites resulting in a decrease in growth. Quin2 is a transition metal ion chelator that potentiates iron-driven oxidant formation. Oxidants were detected in Hepcs buffer. SIN-1 is an oxide-releasing compound and its toxicity to L929 cells is due to a cooperative action of hydrogen peroxide and reactive nitrogen species in the presence of HEPES.

4.4 Light

Fluorescent light can cause the deterioration of tissue culture medium, resulting in toxicity and mutagenicity. The deleterious effects are due to prolonged exposure, whereas short exposures can produce a mitogenic effect. The photo toxicity is due to wavelengths of light that can be absorbed by riboflavin. The pairing of riboflavin with tryptophan, HEPES and guanine results in toxicity mediated by reactive oxygen species. Tryptophan and riboflavin are found in conventional and serum-free optimized media and because they are essential they will be included in all newly designed media. Limited exposure to fluorescent light during manufacture and storage is critical. Specially designed fluorescent tubes not emitting the relevant wavelengths or shields to block those wavelengths are available.

5 Purity of components

In cell culture the largest component of the system is water. Consequently, a relatively small contaminant in the water can have a significant effect on the cells. Conversely water may provide essential nutrients that become limiting as the purity of water is increased. One class of nutrients that could account for this phenomenon is the trace metals. Ideally, in chemically defined media all the components are known and there are no contaminants present. Though the availability of recombinant proteins and peptides has made the pursuit of chemically defined media more attainable, most fall short of this ideal. Cells may need albumin or other serum fractions for the desired performance or stability. Also, manufacturers may not necessarily screen for all potential contaminants in their organic compounds, such as selenium contamination of a thyroxine preparation. The many surfaces with which the medium components come in contact during manufacturing may contribute contaminants. The bioprocessing industry is redefining the notion of purity due to the potential infectious agents that may be present in medium components. It is desirable that all components are of non-animal origin. For example methionine and cysteine are generally isolated from animal hair, so new sources are required.

6 Fatty acids

Serum provides lipids in various forms to cultured cells. These forms include cholesterol, phospholipids, triglycerides, fatty acids, fat soluble vitamins, and various esterified forms of these lipids. The essential fatty acids that cannot be synthesized by animals are 18:2n-6 and 18:3n-3 ('18' refers to the number of carbon atoms and the number following the colon refers to the total number of double bonds in the molecule (Table 1). Animals do not contain the enzymes necessary to place a double bond in these positions. Consequently, the acquisition of these molecules occurs only through an exogenous supply; however, animals (excluding true carnivores) contain desaturation and elongation/shortening enzymes that result in polyunsaturated fatty acids, most notably arachidonic acid, 20:4n-6,

and eicosapentenoic acid, 20:5n-3 and fatty acids of higher degrees of unsaturation and elongation.

Table 1. Fatty acid composition of phosphatidylcholine of normal human fibroblasts supplemented with various fatty acids

Fatty acid[c]	Supplementation[b]		
	18:1n-9	18:2n-6	18:3n-3
16:00	25.80[d]	32.7	33.4[e]
16:1n-9	7.5	5.9	7
16:ln-7	1.8	2.2	3.2
18:00	3.7	7.2	7.3
18:ln-9	48.8	18.1	22.3
18:1n-7	4.6	4	4.3
18:2n-6	0.6	17.2	1.5
18:3n-3	0	0	6
20:ln-9	0.7	0.2	0.1
20:3n-9	1.8	1.4	0.60
20:3n-6	0.2	2.2	0.2
20:4n-6	0.6	4.6	0.7
20:3n-3	0	0	0.9
20:4n-3	0	0	1.9
20:5n-3	0	0	5.3
22:3n-9	0.6	0.2	0.3
22:4n-6	0	0.5	0
22:5n-3	0	0	0.5
22:6n-3	0.4	0.4	0.5
Degrees of saturation[a]			
Saturated	32.2	43.2	44.8
Monounsaturated	63.7	30.4	36.9
Polyunsaturated	4.1	26.5	18.3

[a]This level of FBS was insufficient to supply the required fatty acids for these fibroblasts under these conditions. Consequently, the cells displayed an essential fatty acid deficiency profile (not shown), similar to that displayed by the cells supplemented with the 18:ln-9 fatty acid. Subsequently, the cells were supplemented with pure fatty acids complexed to fatty acid depleted bovine serum albumin for several days.

[b]After the fibroblasts were cultured in an essential fatty acid deficient environment the medium was supplemented for several days with 18:ln-9, 18:2n-6, and 18:3n-3 fatty acids complexed to fatty acid depleted bovine serum albumin. The phospholipids were extracted, phosphatidylcholine purified, and the fatty acids of that phospholipid evaluated as fatty acid methyl esters by GLC.

[c]Fatty acids are designated by the number of carbon atoms (first two numbers), the number of double bonds in the molecule (the number after the colon), and on which carbon atom the first double bond is from the last 'n[th]' carbon (n-position of carbon atom) in the fatty acid molecule, previously named the omega carbon.

^dValues are expressed as the nmole % of total fatty acids.

^eThis represents the sum of the nmole % of the fatty acids for each designation.

     The removal of serum can result in an essential fatty acid deficiency because serum is the predominant lipid source for cultured cells. In the absence of a lipid source cells will synthesize, desaturate, and elongate fatty acids of the n-9 and n-7 families. Under these deficiency conditions there is a characteristic fatty acid profile represented by the polyunsaturated fatty acids of the n-9 and n-7 families. Cultured cells will utilize exogenous fatty acids preferentially to endogenously synthesized ones. The various fatty acid families are metabolized by the same enzymes but at different rates. The essential fatty acids are preferentially metabolized over the non-essential fatty acid families. This situation can be exploited to generate cells with a fatty acid profile favouring a particular fatty acid family (Table 1). Polyunsaturated fatty acids have an extensive metabolism; generating eicosanoids, epoxides with vascular function, anandamide (a ligand of the cannabinoid receptors), and non-enzymatic oxidation products of 20:4n-6, the isoprostanes, which are isomers of prostaglandins. Polyunsaturated fatty acids also have functions that result from the fatty acid and not its metabolic product. For example, intracellular arachidonic acid opened a potassium-selective channel in neonatal rat atrial cells and under some conditions extracellular arachidonic and docosahexanoic (22:6n-3) acids blocked the major voltage-dependent potassium channel (Kv l.5) in cardiac cells. Fatty acids can also influence the physical characteristics of the membrane by influencing its fluidity. Polyunsaturated fatty acids can increase the membrane fluidity, as in J774A.1 cells, and decrease the uptake of acetylated low density lipoprotein. The fatty acid composition of normal human fibroblasts *in vitro* is demonstrated in Table 1. The supplemented fatty acid is represented to a large extent in the phosphatidylcholine (PC) of fibroblasts. The cells are capable of metabolizing the precursor fatty acid by elongation and desaturation to other fatty acids. Cells with different fatty acid compositions may be functionally different.

     It may be tempting to consider the removal of serum the final step in the process of generating a scrum-free medium, but care must be taken to consider the fatty acid composition of the cells.

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Chapter 6
Three-dimensional culture

1 Introduction

Three-dimensional (3D) cell cultures have been widely used in biomedical research because the early decades of this century. Holtfreter and later Moscona pioneered the field by their research on morphogenesis using spherical reaggregated cultures of embryonic or malignant cells. Numerous subsequent *in vitro* studies on organogenesis or expression of malignancy were based on these early investigations. Substantial novel input into research on cell aggregates came from fundamental studies of Sutherland and associates. They pioneered multicellular tumour spheroids (MCTS) as an *in vitro* model for studies on tumour cell response to therapy. MCTS have also been used to study basic biological mechanisms, including the regulation of proliferation, differentiation, cell death, invasion, angiogenesis, and the immune response.

One major advantage of 3D cell cultures is their well-defined geometry-whether planar or spherical-which makes it possible to directly relate structure to function, and which enables theoretical analyses, for example of diffusion fields. Combining such approaches with molecular analysis has demonstrated that, in comparison to conventional cultures, cells in 3D culture more closely resemble the *in vivo* situation with regard to cell shape and cellular environment, and that shape and environment can determine gene expression and the biological behaviour of the cells. One impressive example is the ectopic implantation of embryonic cells, which can result in malignant transformation, whereas the same cells undergo normal embryogenesis in the uterus. Conversely, teratocarcinoma cells may undergo normal development when implanted into an embryo. One further example is the relative resistance of cancer cells to drugs in 3D culture compared to the same cells grown as conventional monolayer or in single cell suspension.

2 Multicellular tumour spheroids (MCTS)
2.1 MCTS monocultures
2.1.1 The history of MCTS and their relevance to solid tumours

Multicellular tumour spheroids (MCTS) mimic solid tumours better than monolayer cultures. MCTS have significantly increased our knowledge of radiation response of mammalian cells, intercellular communication, cell invasion, angiogenesis, and neovascularization. Although attempts to initiate spheroids directly from biopsies for routine individual predictive drug testing had little success, MCTS from established cell lines provide an *in vitro* model to study mechanisms controlling drug penetration, binding, and action. In terms of three-dimensional growth, MCTS behave like the initial, a vascular stages of solid tumours *in vivo,* unvascularized micrometastatic foci, or intercapillary tumour microregions, as shown in Figure 1. Growth kinetics can be described by the Gompertz equation which has been shown to model three-dimensional *in vivo* and *in vitro* growth reasonably well. MCTS develop discrete cell populations similar to those found in microregions of solid tumours with three major groups: actively cycling cells closest to the blood and nutrient supply; quiescent, yet intact and viable cells as intermediates; and necrotic cells/areas furthest from the blood supply (Figure 2). Beyond a critical size (> 500 μm), most spheroids from established cell lines develop massive necrosis in the centre, surrounded by a viable rim of cells with a thickness of 100-300 μm.
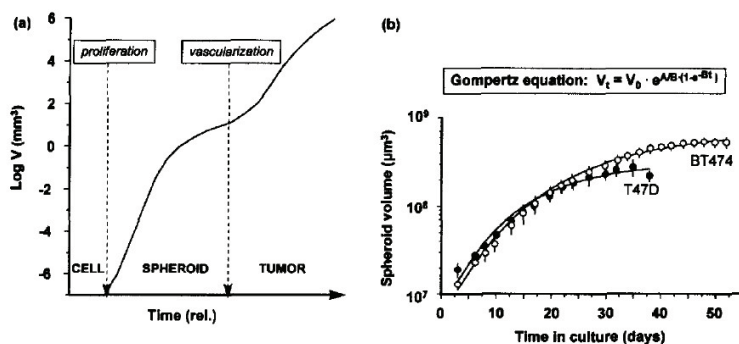
Figure 1. (a) Schematic illustration of the different stages of solid tumour growth that can be mathematically described by the Gompertz function. (b) Representative spheroid volume growth kinetics of two different human breast cancer cell lines grown in liquid-overlay culture in conventional, supplemented media. Lines show non-linear least squares best fits to the Gompertz function with $V_0 = 0.498 \times 10^7$, $A = 0.298$, and $B = 0.060$ for BT474 cells, and $V_0 = 0.578 \times 10^7$, $A = 0.331$ and $B = 0.083$ for T47D cells.
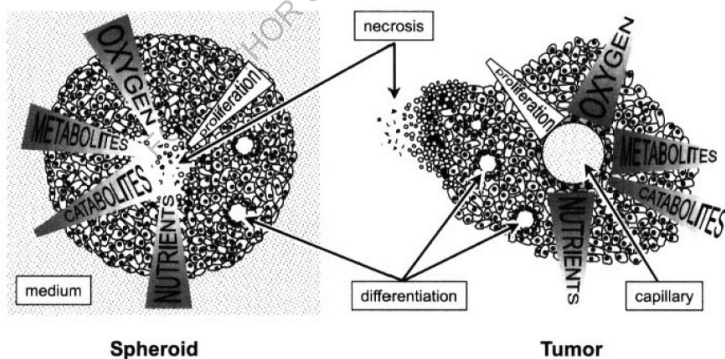
Figure 2. Schematic illustration of the analogy between tumour microregions and multicellular tumour spheroids.

2.1.2 Current research with MCTS monocultures

Many of the early findings with the V79 (Chinese hamster lung cells) and the EMT-6 (mouse sarcoma) spheroid models were supported by subsequent studies with human tumour cell lines. Necrotic cell death in the spheroid centre mainly relates to the limited inward and outward diffusion (Figure 2). Thus, in some tumour spheroid types, such as WiDr (human colon adenocarcinoma) and RatlTl (ras-transfected rat embryo fibroblasts), necrosis and

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hypoxia are coincident and necrotic cell death might be explained by a single limiting factor. In contrast, there is experimental evidence from other MCTS types that necrosis is a complex, multifactorial event. Investigations on microenvironmental and epigenetic mechanisms involved in the regulation of cell proliferation, differentiation, and gene expression have been intensified enormously within the last ten years. Many cell types seem capable of maintaining intracellular homeostasis in 3D culture until shortly before necrotic cell death, despite environmental stress. There are almost no structural or metabolic markers for this 'pre-necrotic' stage except for a reduced oxygen uptake and/or respiratory activity, and a decrease in mitochondrial function. Cell cycle arrest in the nutrient deprived inner regions of large *myc/ras* co-transformed rat fibroblast spheroids (MR1) is accompanied by an upregulation of the CDK inhibitor p21Kipl/Cipl, while cell cycle arrest in monolayer cultures and in myc-transfected, non-tumorigenic aggregates is associated with enhanced expression of pl8lnk4. These data showed for the first time that cell cycle control differs mechanistically in monolayer and spheroid culture and may change at different stages of malignant transformation. Due to the massive central necrotic cell death in MCTS, programmed cell death in 3D aggregates has long been ignored. However, recent data show that apoptosis occurs in spheroids of intestinal epithelial cells. Growth inhibition via stimulation of apoptosis has also been described for p53-defective human lung cancer spheroids transfected *in* situ with a viral wildtype p53 vector. Mueller-Klieser reported apoptotic cell death of multinucleated giant cells in a highly differentiated rhabdomyosarcoma spheroid and in 3D cultures of V79 cells using the TUNEL assay. 3D cultures undergo morphological and functional reorganization. Amongst these activites are:

(a) Modified deposition and/or assembly of bio matrix such as the extracellular matrix molecules fibre nectin, collagen types I, III, and IV or laminin.
(b) Expression and function of ECM receptors (integrins), receptor subsets and subunits and cadherins.
(c) The expression and function of biological response modifiers such as growth and angiogenic factors (i.e., EGF, TGFcc, VEGF) and/or their receptors.
(d) The development of gap junctional communication.

2.1.3 MCTS culture

A common principle of all spheroid culture methods is the prevention of cellular attachment to a substrate such as surfaces of Petri dishes. Many non-transformed and most malignant cells tend to form spherical aggregates under these conditions. There are two strategies for cultivating MCTS: in stirred medium and in static medium.

The most widely used method for growing MCTS from established cell lines is the spinner flask culture (Figure 3). With this technique a large number of MCTS may be generated simultaneously in large volume cultures, and these can be scaled-up to mass culture. While some tumour cell types are capable of producing cell aggregates directly from single cell suspensions in spinner flasks, cell-cell interaction, and thus aggregate formation, is often more efficient under static conditions. As a consequence, bacteriological Petri dishes or agar/agarose-coated culture dishes, to which cells do not adhere, are often used to initiate spheroid formation.

Cell number and initiation period are critical variables which depend on the cell type and culture conditions (medium, serum pH, etc.). In general, the cell number inoculated varies between 1×10^5 and 2×10^6 exponentially growing cells per 100 mm dish. Once aggregates have emerged in the unstirred medium within two to five days, they can be

transferred into spinner flasks. Medium is not refreshed during the initiation period, whereas it is renewed routinely in spinner cultures. As spheroid initiation may lead to the formation of aggregates with a large variability in size, and as spheroid-to-spheroid clusters may occur, selection of a specific spheroid population before the transfer into spinner flask is recommended. If several selective sedimentation steps, for example using Falcon tubes, are not sufficient to eliminate large cell clusters and single cells, hand-selection or rapid harvesting of spheroids by sieving through nylon screens can be performed. The coefficient of variation of the average spheroid diameter within the selected spheroid population should be other frequently used techniques to generate large numbers of spheroids are roller tubes and the 'gyratory shaker' method shown in Figure 3. As for spinner flask cultures, an initiation period under non-stirring conditions may be required and the medium should be renewed routinely after transfer into the roller tubes or onto the gyratory shaker. Also, to guarantee optimum constant supply conditions throughout spheroid growth, the number of cells per volume of medium should be kept relatively constant by gradually reducing the number of spheroids per flask. For many tumour cell types, the optimum cell number does not extend beyond 3×10^5 cells per ml of medium, and two thirds of the medium has to be refreshed every 24-48 hours.

The most convenient method of growing spinner flask cultures is in a humidified CO_2 incubator at 37 °C, if a stirring device suited for incubator atmospheres is available. Alternatively, spinner flasks may be sealed and kept in a non-humidified, temperature controlled atmosphere or in a thermostatically controlled water-bath positioned over a magnetic stirrer. If the volumes of the gas phase and of the culture medium are approximately the same, the medium of the sealed cultures may be refreshed every 24 hours without generating unfavourable environmental conditions due to cellular production of CO_2 and metabolic waste. The size of commercially available spinner flasks for research purposes ranges from 100 ml to 1 litre with a recommended maximum filling level of approximately 50% of the total volume. The choice of size should be determined by the number of spheroids needed or the (minimum) volume of supplemented medium to be applied. Different types of spinner flasks are available from various companies. While the handling of these spinner flasks, in particular the effort required for cleaning, may differ, the authors have experienced no relevant difference between them in the growth kinetics of MCTS. An additional parameter that should be optimized is the stirring frequency and thus the shear force in the spinner culture. Optimization should minimize the number of cells that are shed from the spheroids into the medium. Some cell types exhibit an intrinsically high cell shedding in spheroid culture that may not be decreased by the growth conditions. As a result, shed single cells may form small clusters and/or adhere to the vessel wall. In general, these complications can be alleviated, if not eliminated, by changing the culture medium more frequently and by siliconization of the inner surfaces of the spinner flasks. Some cell types do not form ideally spherical spheroids. Indeed, some rumour cells do not even exhibit cell aggregation, such as some variants of the MCF-7 breast cancer cell line. If no or only irregular cell clusters are obtained, a number of parameters can be modified to achieve satisfactory cell-cell aggregation and aggregate growth, including:

(a) Initiation of aggregate formation:
- serum type (batch)
- serum content
- culture medium (glucose and glutamine concentrations)
- non-adhesive dish (microbiological, agar-coated)
- CO_2 in incubator (pH in culture medium)

- cell concentration

(b) Initial phase of spinner flask culturing:
- same factors as in (a); each factor may be different from those in (a)
- stirring frequency
- spinner flask geometry

Spheroid Culturing Techniques
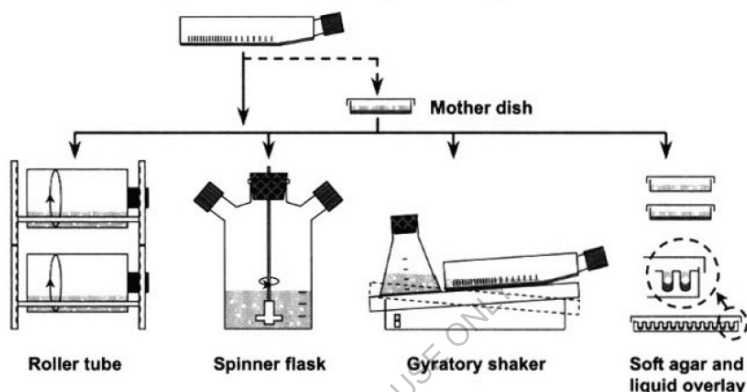
Figure 3. Methods for the cultivation of multicellular spheroids under continuous stirring (spinner flash, roller tube, gyratory shaker, etc.) or static on non-adherent surfaces (liquidoverlay in 96-well plates).

Spinner culture is preferable to growth of MCTS on stationary, non-adhesive surfaces, if large numbers of spheroids with a maximum size are needed. Spheroids continuously grown on agar/agarose-coated surfaces may not reach their maximum size, and the thickness of the viable cell rim is often relatively small. Conversely, various modifications for culturing spheroids in socalled agar- or liquid-overlay (figure 3) have been established. The application of 96-well plates is of particular interest, because individual spheroids can be monitored repeatedly and may be manipulated throughout growth. Following an initiation period under static conditions, liquid-overlay cultures may be kept on a gyratory shaker to guarantee optimum nutrient supply. As a prerequisite, 96-well plates are coated with 0.5-1.5% agar/agarose. A novel semi-adhesive substrate used for spheroid cultivation takes advantage of the spontaneous detachment of small cell aggregates formed on semi-adhesive substrates such as proteoglycans or positively charged polystyrene.

2.1.4 Additional methodological aspects

In addition to the volume of the spheroids, which should be monitored routinely, analysis of the proliferative activity is useful. The proliferation status can be determined autoradiographically by [^{3}H] thymidine labelling (TLI = thymidine labelling index) or immunohistologically utilizing the BrdUrd antibody technique or antibodies detecting specific proliferation-associated antigens such as PCNA or Ki67. Alternatively, spheroids may be dissociated enzymatically and fixed or unfixed single cell suspensions can be analysed for their cell cycle distribution using flow cytometry and DNA-specific

fluorochromes such as propidium iodide, 7-AAD (7-aminoactinornycin D), mithramycin, or Hoechst DNA dyes. Sections of frozen or fixed, paraffin-embedded spheroids can be stained by any conventional labelling technique, or examined by scanning or transmission electron microscopy.

A method for isolating specific cell subpopulations from spheroids is described in detail. This automated dissociation technique is based on the sequential exposure of the cell aggregates to an enzyme solution. The experimental set-up consists of autoclavable, standard laboratory equipment mounted on a gyratory platform in a 37°C water-bath as shown in Figure 4. The enzyme solution is continuously pumped through a temperature equilibration coil into a 'racetrack' dissociation chamber which is filled with 15 ml of enzyme solution before the dissociation procedure. The chamber consists of two compartments which are separated by a nylon screen with a pore size of 75 um. Pumping enzyme solution into the outer compartment and out of the inner part generates a flow of liquid through the screen. Thus, the spheroids are kept in suspension in the outer compartment, while dissociated single cells reach the inner compartment, following the fluid flow. The cell suspension is constantly removed from the inner compartment and collected on ice. Sequential aliquots are sampled from different levels within the spheroids.
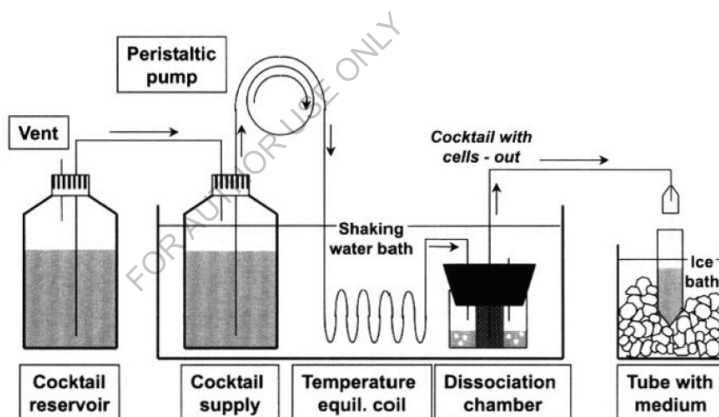
Figure 4. Experimental set-up for automated selective dissociation of multicellular spheroids consisting of an enzyme cocktail reservoir and supply unit, a peristaltic pump for enzyme transport through a temperature equilibration coil into a specially designed dissociation chamber, both placed in a 37°C temperature controlled shaking water-bath. The dissociation chamber contains an outer and an inner chamber which is separated by a 75 μm mesh, and is sealed by a stopper. Spheroids are placed in the outer chamber and the enzyme cocktail is pumped through the chamber so that dissociated cells are transferred into a tube containing ice-cold medium.

2.2 MCTS co-cultures
2.2.1 General aspects of tumour heterogeneity

The presence of histologically different cell types within tumours, including host defence cells, fibroblasts and endothelium contributes to their heterogeneity. This heterogeneity can to some extent be mimicked *in vitro* by heterologous spheroids consisting of rumour cells and host cells. Such spheroids may be initiated directly from mixtures of suspended host cells, such as fibroblasts or monocytes and Tumour cells. One disadvantage of this approach is the risk of non-reproducible formation of intraspheroidal clusters of one cell type. Alternatively, preformed MCTS of defined sizes and histomorphological characteristics can be incubated with suspensions of immune cells such as monocytes or LAK (lymphokine-activated killer) cells, to study immune cell infiltration and its cytostatic and/or cytotoxic effects on the tumour cells.

2.2.2 Co-cultures of MCTS and immune cells

Mononuclear cells from peripheral blood can be prepared by standard density centrifugation over Ficoll/Hypaque, and human blood monocytes can be separated from this cell mixture by their intrinsic activity to adhere to plastic or glass surfaces. A more convenient technique uses leukaphoresis and countercurrent elutriation. Differentiated macrophages can be generated by long term culture in Teflon bags, and these can be used for co-cultivation experiments.

2.2.3 Co-cultures of MCTS and flbroblast aggregates

The extracellular matrix (ECM), mainly produced by stromal fibroblasts, has a crucial role in epithelial cell differentiation, growth, and gene expression. To investigate interactions between stromal fibroblasts and tumour cells, MCTS can be cultured on confluent fibroblast monolayers or with fibroblast aggregates as described with human bladder and breast cancer cells. While some tumour cell types overgrow fibroblast aggregates and may show some specific cell-cell interactions in the contact zone, others, in particular highly metastatic tumour cells, seem to invade the fibroblast aggregates.

2.2.4 Co-cultures of MCTS and endothelial cells

MCTS are also used in studies of angiogenesis and invasion, such as the embryonic chick heart fragment confrontation culture system in semi-solid medium. Culture of MCTS on confluent endothelial cells with underlying ECM can be used to study tumour cell invasion, but not angiogenesis. Data obtained with melanoma cell spheroids have led to the hypothesis that free radical-mediated endothelial cell damage is one mechanism contributing to melanoma metastasis.

2.2.5 Additional technical aspects

Separation of cells from defined locations within sections from 3D cultures can be achieved using microdissection techniques, such as laser microbeam combined with an optical tweezer. Dissociation of co-cultures by enzymatic or mechanical means allows for flow cytometric analyses and for separation of cellular subpopulations by various sorting techniques. As a prerequisite for cell detection and separation, cell subpopulations can be permanently labelled by the introduction of specific marker genes, for example using p-

galactosidase or green fluorescent protein (GFP) or labels for cell membranes. GFP can be detected in routine flow cytometry.

3 Experimental tissue modelling
3.1 Current research on tissue modelling

Substantial progress has been made towards the development of an artificial liver. Strategies include culture of hepatocyte spheroids or of heterospheroids consisting of hepatocytes and fibroblasts or parenchymal cells. The application of artificial support systems, such as porous gelatin sponges, agarose or collagen as well as the induction of aggregation by exogenous adhesion molecules is advantageous for long-term culture of liver cells. Reconstitution of the specific geometry, microenvironment and metabolism of the hepatic sinusoid in a complex 3D culture system has been reported and pig hepatocytes have been grown in co-culture in an artificial 3D capillary system.

Development of an artificial pancreas has focused on spheroids from insulins ecreting recombinant mouse pituitary AtT 20 cells and mouse insulinoma beta TC3 cells. Long-term viability and function of human fetal islet-like cell clusters implanted under the kidney capsule of athymic mice have been achieved. Another promising strategy is the use of isolated piscine islets, so called Brockmann bodies, as 'natural' pancreatic spheroids which can be implanted as xenografts after microencapsulation with alginate. Oxygen supply seems to play a crucial role for the survival and maintenance of function of such islets.

The pituitary gland has been modelled using multicellular spheroids to study hormone release, including luteinising hormone (LH), following stimulation with the releasing hormone LHRH. In contrast to pineal cell monolayers, 3D cultures remain functional for more than three weeks secreting melatonin when challenged with isoproterenol. Despite their tendency to spontaneously form follicles in aggregation culture, there are few reports on isolated adult thyroid cells. Thyroid cell spheroids allow study of cell motility, cell adhesion, and E-cadherin expression in thyroid follicle biogenesis. 3D brain cultures has been reviewed comprehensively and have been used to study neural myelination and demyelination, neuronal degeneration and the neurotoxicity of lead. Neuronal behaviour following HIV infection, Alzheimer's disease, and Morbus Parkinson has been studied using aggregated brain cell cultures. Retinal development has also been modelled. 3D chondrocyte research has focused on molecular aspects of matrix formation and differentiation.

Aggregated cell cultures of heart cells have been used to study aspects of cardiac development and physiology, including sinoatrial node cell preparations, atrial cell preparations, embryonic chick heart cell aggregates and ventricular cell aggregates. Other tissues that have been modelled in 3D cultures include mesangial cells, urothelial cells and nasopharyngeal cells.

3.2 Tissue modelling of skin and mucosa

Heterotypic 3D cultures including fibroblasts and keratinocytes have been used for studies on the formation of skin and hair follicles and the influence of mesenchyme on the differentiation of keratinocytes.

3.3 Embryoid bodies

Embryoid bodies (EB) are derived from embryonic stem cell (ES) cell lines which have retained their capacity to generate cells of the haematopoietic, endothelial, muscle and neuronal lineages. Development of skeletal muscle myocytes in embryoid bodies is similar to that *in vivo* and beating EB with cardie-specific receptors, ionic channels and action potentials have been produced. Oxygen-regulated gene expression during embryonic development has also been studied.

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Chapter 7

Biomaterial Science

1. Introduction to Biomaterials

The development of biomaterials is not a new area of science, having existed for around half a century. The study of biomaterials is called biomaterials science. It is a provocative field of science, having experienced steady and strong growth over its history, with many companies investing large amounts of money into the development of new products. Biomaterials science encompasses elements of medicine, biology, chemistry, tissue engineering and materials science.

"A biomaterial is any material, natural or man-made, that comprises whole or part of a living structure or biomedical device which performs, augments, or replaces a natural function". "A biomaterial is a nonviable material used in medical device, so it's intended to interact with a biological system".

Biomaterials are materials (synthetic and natural; solid and sometimes liquid) that are used in medical devices or in contact with biological systems. Biomaterials as a field has seen steady growth over its approximately half century of existence and uses ideas from medicine, biology, chemistry, materials science and engineering. There is also a powerful human side to biomaterials that considers ethics, law and the health care delivery system. Although biomaterials are primarily used for medical applications, they are also used to grow cells in culture, to assay for blood proteins in the clinical laboratory, in processing biomolecules in biotechnology, for fertility regulation implants in cattle, in diagnostic gene arrays, in the aquaculture of oysters and for investigational cell-silicon "biochips." The commonality of these applications is the interaction between biological systems and synthetic or modified natural materials.

Biomaterials are rarely used on their own but are more commonly integrated into devices or implants. Thus, the subject cannot be explored without also considering biomedical devices and the biological response to them.

Biomaterials can be metals, ceramics, polymers, glasses, carbons, and composite materials. Such materials are used as molded or machined parts, coatings, fibers, films, foams and fabrics.

The definition of a biomaterial does not just include man-made materials which are constructed of metals or ceramics. A biomaterial may also be an autograft, allograft or xenograft used as a transplant material.

Applications

Biomaterials are used in:

- Joint replacements
- Bone plates
- Bone Cement
- Artificial ligaments and tendons
- Dental implants for tooth fixation
- Blood vessel prostheses
- Heart valves
- Skin repair devices
- Cochlear replacements
- Contact lenses
- Breast implants

2. Subjects important to Biomaterials Science

Toxicology

A biomaterial should not be toxic, unless it is specifically engineered for such requirements (for example, a "smart bomb" drug delivery system that targets cancer cells and destroys them). Since the nontoxic requirement is the norm, toxicology for biomaterials has evolved into a sophisticated science. It deals with the substances that migrate out of biomaterials. For example, for polymers, many low-molecular-weight "leachables" exhibit some level of physiologic activity and cell toxicity. It is reasonable to say that a biomaterial should not give off anything from its mass unless it is specifically designed to do so. Toxicology also deals with methods to evaluate how well this design criterion is met when a new biomaterial is under development.

Biocompatibility

The understanding and measurement of biocompatibility is unique to biomaterials science. Unfortunately, we do not have precise definitions or accurate measurements of biocompatibility. More often than not, biocompatibility is defined in terms of performance or success at a specific task. Thus, for a patient who is doing well with an implanted Dacron fabric vascular prosthesis, few would argue that this prosthesis is not "biocompatible." However, the prosthesis probably did not re-cellularize (though it was designed to do so) and also can throw off blood clots (emboli), though the emboli in this case usually have little clinical consequence. This operational definition of biocompatible ("the patient is alive so it must be biocompatible") offers us little insight in designing new or improved vascular prostheses. It is probable that biocompatibility may one day be defined for applications in soft tissue, hard tissue, and the cardiovascular system (blood compatibility).

Functional Tissue Structure and Pathobiology

Biomaterials incorporated into medical devices are implanted into tissues and organs. Therefore, the key principles governing the structure of normal and abnormal cells, tissues and organs, the techniques by which the structure and function of normal and abnormal tissue are studied, and the fundamental mechanisms of disease processes are critical considerations to workers in the field.

Healing

Special processes are invoked when a material or device heals in the body. Injury to tissue will stimulate the well-defined inflammatory reaction sequence that leads to healing. Where a foreign body (e.g., an implant) is present in the wound site (surgical incision), the reaction sequence is referred to as the "foreign body reaction." The normal response of the body will be modulated because of the solid implant. Furthermore, this reaction will differ in intensity and duration depending upon the anatomical site involved. An understanding of how a foreign object alters the normal inflammatory reaction sequence is an important concern for the biomaterials scientist.

Dependence on Specific Anatomical Sites of Implantation

Consideration of the anatomical site of an implant is essential. An intraocular lens may go into the lens capsule or the anterior chamber of the eye. A hip joint will be implanted in bone across an articulating joint space. A heart valve will be sutured into cardiac muscle and will contact both soft tissue and blood. A catheter may be placed in an artery, a vein or the urinary tract. Each of these sites challenges the biomedical device designer with special requirements for geometry, size, mechanical properties, and bioresponses.

Mechanical and Performance Requirements

Biomaterials and devices have mechanical and performance requirements that originate from the physical (bulk) properties of the material. There are three categories of such requirements: mechanical performance, mechanical durability and physical properties. First, consider mechanical performance. A hip prosthesis must be strong and rigid. A tendon material must be strong and flexible. A heart valve leaflet must be flexible and tough. A dialysis membrane must be strong and flexible, but not elastomeric. An articular cartilage substitute must be soft and elastomeric. Then, we must address mechanical durability. A catheter may only have to perform for 3 days. A bone plate may fulfill its function in 6 months or longer. A leaflet in a heart valve must flex 60 times per minute without tearing for the lifetime of the patient (realistically, at least for 10 or more years). A hip joint must not fail under heavy loads for more than 10 years. The bulk physical properties will also address other aspects of performance. The dialysis membrane has a specified permeability, the articular cup of the hip joint must have high lubricity, and the intraocular lens has clarity and refraction requirements. To meet these requirements, design principles from physics, chemistry, mechanical engineering, chemical engineering, and materials science are invoked.

Industrial Involvement

A significant basic research effort is now under way to understand how biomaterials function and how to optimize them. At the same time, companies are producing implants for use in humans and, appropriate to the mission of a company, earning profits on the sale of medical devices. Thus, although we are now only learning about the fundamentals of biointeraction, we manufacture and implant millions of devices in humans. How is this dichotomy explained? Basically, as a result of considerable experience we now have a set of materials that performs satisfactorily in the body. The medical practitioner can use them with reasonable confidence, and the performance in the patient is largely acceptable. Though the devices and materials are far from perfect, the complications associated with the devices are less than the complications of the original diseases.

The complex balance between the desire to alleviate suffering and death, the excitement of new scientific ideas, the corporate imperative to turn a profit, the risk/benefit relationship and the mandate of the regulatory agencies to protect the public forces us to consider the needs of many constituencies. Obviously, ethical concerns enter into the picture. Also, companies have large investments in the manufacture, quality control, clinical testing, regulatory clearance, and distribution of medical devices. How much of an advantage (for the company and the patient) will be realized in introducing an improved device? The improved device may indeed work better for the patient. However, the company will incur a large expense that will be perceived by the stockholders as reduced profits. Moreover, product liability issues are a major concern of manufacturers. The industrial side of the biomaterials field raises questions about the ethics of withholding improved devices from people who need them, the market share advantages of having a better product, and the gargantuan costs (possibly non-recoverable) of introducing a new product into the medical marketplace. If companies did not have the profit incentive, would there be any medical devices, let alone improved ones, available for clinical application?

When the industrial segment of the biomaterials field is examined, we see other essential contributions to our field. Industry deals well with technologies such as packaging, sterilization, storage, distribution and quality control and analysis. These subjects are specialized technologies, often ignored by academic researchers. Also, many companies support in-house basic research laboratories and contribute directly to the fundamental study of biomaterials.

Ethics

A wide range of ethical considerations impact biomaterials science. Like most ethical questions, an absolute answer may be difficult to come by. Some articles have addressed ethical questions in biomaterials and debated the important points (Saha and Saha, 1987; Schiedermayer and Shapiro, 1989).

Regulation

The consumer (the patient) demands safe medical devices. To prevent inadequately tested devices and materials from coming on the market, and to screen out individuals clearly unqualified to produce biomaterials, the United States government has evolved a complex regulatory system administered by the US Food and Drug Administration (FDA). Most nations of the world have similar medical device regulatory bodies. The International Standards Organization (ISO) has introduced international standards for the world community. Obviously, a substantial base of biomaterials knowledge went into establishing these standards. The costs to comply with the standards and to implement materials, biological, and clinical testing are enormous. Introducing a new biomedical device to the market requires a regulatory investment of tens of millions of dollars. Are the regulations and standards truly addressing the safety issues? Is the cost of regulation inflating the cost of health care and preventing improved devices from reaching those who need them? Under this regulation topic, we see the intersection of all the players in the biomaterials community: government, industry, ethics, and basic science. The answers are not simple, but the problems must be addressed every day.

3. Synthetic Polymers as Biomaterials

A synthetic polymer is a long-chain compound derived by bonding together many single unit molecules. The single-unit molecules that constitute a polymer are known as

monomers. Examples of synthetic polymers include non biodegradable polymers, such as polyethylene, poly (vinyl chloride), Dacron, nylon, and Teflon, and biodegradable polymers, such as polyglycolides, polylactides, polyanhydrides, and polysaccharides. Synthetic polymers possess unique features suitable for biological applications. These materials can be modulated to change their chemical and mechanical properties, and can be tailored into various shapes. In addition, these materials are light, strong, and inert. Thus, synthetic polymeric materials have been widely used for the repair, replacement, and regeneration of injured and disordered tissues. In this section, the classification, structure, and properties of synthetic polymers as well as their application to tissue engineering are discussed.

Classification

Polymers can be classified into two groups according to on the mechanisms of synthesis: addition polymers and condensation polymers. An *addition polymer* is synthesized by connecting monomer units via rearranging chemical bonds. The formation of poly (vinyl chloride) is a typical example of addition polymer. In this case, the double bond of a single vinyl chloride opens up in the presence of an initiator such as a peroxide molecule. The initiator can be activated by increasing temperature or exposure to ultraviolet. The initiator can activate monomers to form free bonds, which connect the monomers together. This process is known as propagation. Common addition polymers include polyethylene, polypropylene, polystyrene, and poly (vinyl chloride).

A *condensation polymer* is synthesized by joining two molecules together with the elimination of a molecule such as water and methanol. Two different types of monomers are usually participating in the polymerization reaction. The resulting product is known as copolymer. Nylon is a typical copolymer. During the polymerization process of nylon, a dicarboxylic acid molecule reacts with a diamine. A water molecule is removed when the two molecules are bonded. Examples of condensation polymers include polysaccharides, proteins, polyesters, polyamides, polyurea, polyurethane, and cellulose.

Based on the degradability of polymeric materials in a biological system, polymers can be classified into non-biodegradable and biodegradable polymers. Non biodegradable polymers cannot be degraded, whereas biodegradable polymers can be degraded in a biological system. Examples of nonbiodegradable polymers used as biomaterials include polyethylene, polytetrafluoroethylene, poly (vinyl chloride), and polypropylene. Examples of biodegradable polymers include polyglycolides, polylactides, polysaccharides, and poly (α-hydroxyl acids). In terms of the type of monomer in a polymer structure, polymers can be classified into homopolymers and heteropolymers (or copolymers). Homopolymers are compounds constituted with one type of repeated monomers. Examples of homopolymers include polyethylene, polytetrafluoroethylene, poly (vinyl chloride), and polypropylene. Copolymers are polymeric compounds composed of two or more types of monomer. The different monomers may be randomly distributed or may alternate in a pattern. Examples of copolymers include poly (glycolide lactide), polyurethane, and poly (glycolide trimethylene carbonate).

General Properties

Polymeric materials exist in several forms, including liquid, elastomer, and plastic. The form of polymeric materials is determined by a number of factors, including the nature of monomers, the size and form of polymer molecules, the type and concentration of catalysts

used for polymer synthesis, and curing temperature and duration. By selectively altering these factors, a desired form of polymeric materials can be generated. An important factor that influences the structure and mechanical properties, such as flexibility and strength, of polymeric materials is the composition of polymer molecules. A substitution of a key atom in a monomer may induce a significant change in the mechanical properties. For instance, the replacement of the carbon atom with oxygen in a polyethylene molecule reduces the rigidity of the polymer.

The molecular size or polymeric chain length is another critical factor that determines the structure and mechanical properties of polymeric materials. Polymers are composed of various numbers of monomers. For the same type of monomer, a longer polymer is more flexible and tangled more easily than a shorter one. For instance, polyethylene and paraffin can be synthesized based on the same type of monomer CH_2CH_2, but with different chain length. The length of a paraffin molecule is much shorter than that of a polyethylene molecule. The long-chained polyethylene molecules are more difficult to crystallize, thus exhibiting higher flexibility, compared to paraffin. The chain length of polymers is one of the factors that determines the rigidity and strength of polymer materials. An increase in the chain length reduces the mobility of polymer molecules, and thus enhances the rigidity and strength. The form of polymer molecules is a major factor that influences the structure and organization of polymeric materials. Polymer molecules exit in several forms: linear, branched, and cross linked forms. A linear polymer molecule is composed of a single chain of monomers with various lengths and molecular weights. Examples of linear polymers include polyvinyls and polyesters. These molecules can be partially crystallized to form so-called semicrystalline polymers. It is usually difficult to completely crystallize these polymers.

A branched polymer molecule is composed of a main chain and various densities of side chains. Copolymerization may enhance the formation of branches. Since the side branches influence the interaction between the main polymer chains, the introduction of side branches may reduce polymer crystallization, yielding more flexible polymers. An increase in the length of the side chain reduces the melting temperature of the polymer in association with a reduction in crystallization.

Polymer molecules can be cross linked to form networks. Long-chain polymer molecules are usually linked together with side chains. Cross linked polymers are usually difficult to crystallize. An increase in the degree of cross linking reduces the crystallization capability of polymeric materials. Up to a certain degree of cross linking, polymer crystallization may be completely prevented. During the cross linking process, the form of monomers and reaction conditions may influence the structure and mechanical properties of the polymeric material. For instance, the presence of tortuous and curved polymer chains may result in a polymeric material that is elastic and flexible. This type of material can be stretched to a large extent, and can return back to the undeformed length upon the release of the stretching force. Natural rubber (*cis*-polyisoprene) is a typical example of such materials.

In addition to the intrinsic factors of polymeric molecules, environmental factors influence the structure and mechanical properties of polymeric materials. A typical environmental factor is temperature. An increase in temperature often reduces the rigidity of polymeric materials and induces the transformation of polymer from plastic to elastomer and liquid.

4. Nonbiodegradable Polymers

Polytetrafluoroethylene

Polytetrafluoroethylene (PTFE) is a fluorocarbon polymer, also known as Teflon, which is commonly used as a biomaterial for the replacement of soft tissues. Teflon is characterized by high crystallization (about 90% molecules crystallized), a relatively high density ($\sim$2 g/cm^3), and low surface tension and friction compared to other types of polymer. Teflon is used primarily for the construction of vascular substitutes. Teflon vascular grafts are sufficiently strong for withstanding stretching forces induced by arterial blood pressure. The mechanical properties of Teflon grafts can be stable for years following implantation. Because Teflon stimulates inflammatory reactions and thrombogenesis, which lead to the development of intimal hyperplasia, it can only be used for the replacement of arteries larger than 4 mm in diameter. Teflon can also be used to construct supporting sheaths for vein grafts, an approach for modulating the diameter of the vein graft, reducing diameter mismatch-induced disturbance of blood flow, and suppressing flow disturbance-induced intimal hyperplasia.

Poly (ethylene terephthalate)

Poly (ethylene terephthalate) (PET), known as Dacron, is a polyester material that is characterized by hydrophobicity, resistance to hydrolysis, and high strength and toughness. Dacron has primarily been used for the construction of vascular grafts. Porous grafts can be constructed by weaving PET fibres into mesh-like materials. This type of graft usually facilitates cell integration into the graft wall when anastomosed into a host artery. Dacron grafts are often used to replace malfunctioned thoracic and abdominal aortae. As other polymeric materials, Dacron induces inflammatory reactions and thrombogenesis. Thus, this type of graft can only be applied to arteries with diameter exceeding 4 mm.

Polyethylene

Polyethylene is a polymer composed of ethylene monomers and can be synthesized into polymeric materials of various densities. A low-density polyethylene material (0.91–0.93 g/cm^3) can be synthesized by using peroxide catalysts at pressure 1000–3000 kg/cm^2 and temperature 300–500ºC. Polyethylene materials generated under such conditions are composed of branched polymers that are difficult to crystallize. This type of material is tough and flexible, and is often used to fabricate thin membranes for food packaging and also for manufacturing biomedical supplies, such as tubing and containers.

A high-density polyethylene material (0.945–0.96 g/cm^3) can be synthesized by using metal catalysts at pressure $\sim$10 kg/cm^2 and temperature 60–80ºC. This polymer material is composed primarily linear polymers and is highly crystallized with strong bonds between ethylene monomers. The high-density polyethylene material is stronger than the low-density polyethylene material, and has been used for fabricating orthopaedic implants, such as load-bearing caps for artificial joints.

5. Biodegradable Polymers

A number of biodegradable polymeric materials have been synthesized and used as biomaterials. These materials belong to several polymer families, including linear aliphatic

polyesters, polyorthoesters, polyphosphate esters, poly (ester–ether), polyanhydrides, polyamides, polysaccharides, polyamino acids, and inorganic polyphosphazenes. Some of these polymers have been increasingly used for the regeneration, repair, and replacement of tissues and organs. Because these materials can be gradually degraded and removed through various organ systems such as the liver and kidneys, harmful influences, if any, imposed by these materials can be eliminated. Furthermore, when used for constructing tissue scaffolds, biodegradable polymers with desired shapes can serve as guidance for cell migration and pattern formation. With the degradation of the polymer scaffold, natural tissues can be gradually established and strengthened, eventually integrating into the host system.

Biodegradable polymeric materials have been applied to biomedical research in primarily two areas: wound healing and drug delivery. Linear aliphatic polyesters, such as polyglycolides, poly (glycolide-l-lactide), poly (ester–ether), and poly (glycolide–trimethylene carbonate), have been successfully used for enhancing wound closure and healing. These polymers have been extensively studied for their structure, material properties, and biological compatibility. Several types of biodegradable polymer have been used for constructing drug delivery carriers. Examples include polyanhydrides and poly (ester–ether). By controlling the rate of degradation, the rate of drug delivery can be regulated. In addition, biodegradable polymers have been investigated for their potential use in tissue regeneration and repair. For the last decade (since the mid-1990s), the synthesis and characterization of biodegradable polymers are among the most active research areas in biomedical engineering. Here, several common types of biodegradable polymers are discussed with a focus on the structure, material properties, and potential applications to bioregenerative engineering.

Linear Aliphatic Polyesters

Linear aliphatic polyesters are straight-chain polymers in which monomers are joined by the ester bond —COO—. Commonly used linear aliphatic polyesters include polyglycolide (PG) or poly (glycolic acid) (PGA), polylactide (PL) or poly(lactic acid) (PLA), polycaprolactone (PCL), poly(β-hydroxybutyrate), and poly(glycolide–trimethylene carbonate). These polymers have been successfully used in biomedical research and application. Among these aliphatic polymers, polyglycolide and polylactide are basic molecules that can be used to form copolymers and derive different forms of polymers. These polymers have been frequently used in biomedical research.

Polyglycolides and Polylactides

Polyglycolides and *polylactides* can be synthesized from glycolic acids and lactic acids, respectively. The direct condensation method can be used to synthesize low-molecular-weight polyglycolides or polylactides (<3000 kDa). Common catalysts for such synthesis include phosphoric acids, *p*-toluene sulphonic acid, and antimony trifluoride. For a polymer larger than 3000 kDa, a process known as *ring opening polymerization* is necessary for polymer synthesis. Several catalysts, such as stannous chloride dehydrate and aluminum alkoxide, has been used for the synthesis of large polyglycolides and polylactides. The thermal properties of polymers are described by the melting and glass transition temperature, while the mechanical properties of polymers are expressed by the elastic modulus, tensile strength, and maximal distensibility or strain. A typical polyglycolide material has a melting

temperature of ~210ºC and a glass transition temperature of ~36ºC, whereas a polylactide material possesses a melting temperature of ~170ºC and a glass transition temperature of ~56ºC. Mechanically, polylactide-based materials possess elastic moduli ranging from 1200 to 3000 MPa and tensile strength of 28–50 MPa depending on the molecular weight, and can be extended to 2–6% of their original length before reaching the breakpoint.

The rate of polymer biodegradation is an important parameter considered in the design and synthesis of biodegradable polymers. Polymers are usually degraded by hydrolysis of the ester bonds, resulting in a decrease in molecular weight. Several factors are known to influence the rate of polymer degradation. These include the chemical structure and molecular weight of the polymer material as well as environmental conditions. For instance, amorphous polymers can degrade more easily than crystalline polymers. Polymers with a higher molecular weight may be degraded more slowly than those with a lower molecular weight. Branched polymer molecules may be degraded faster than linear molecules. An increase in temperature facilitates polymer degradation. For a typical semicrystalline polylactide material, weight loss can be detected after 30 weeks in a phosphate buffer at 37ºC.

Glycolides and lactides can form copolymers with each other as well as with other types of esters. The copolymer formed on the basis of glycolides and l-lactides is known as poly (glycolide-l-lactide). The relative contents of various esters in a copolymer may influence the rate of polymer degradation. For instance, the degradation rate of poly (glycolide-l-lactide) is dependent on the relative concentration of lactides. It is interesting to note that the relationship is not linear. In the range of 0–25% of l-lactides, the rate of degradation is inversely proportional to the concentration of l-lactides, whereas in the range of 75–100% of l-lactides, the rate of degradation is directly proportional to the concentration of lactides. Interestingly, in the range of 25–75% of l-lactides, the rate of polymer degradation does not change significantly with an increase in the concentration of lactides. Thus, for the design of a copolymer, the degradation rate should be taken into account. Ideally, a polymer material should have a degradation rate that is comparable to the rate of native tissue formation.

Polyglycolides, polylactides, and their copolymers have been used for constructing various forms of matrix for biomedical applications such as drug delivery and tissue repair. These polymers can be also injected into target tissues. The biocompatibility and toxicity of these polymers have been tested extensively. The degradation product of polylactides is lactic acid, which is a natural metabolite in mammals and can be removed via physiological metabolism. The metabolite of polyglycolides, glycolic acid, has been shown to be a low-toxicity substance. An increase in acidity near a polymer implant may occur, but such a change can be mitigated by a local pH treatment. In general, these polymers exhibit low toxicity and are safe for *in vivo* implantation and injection.

Polycaprolactones

Polycaprolactone (PCL) are another type of polyester that is used in biomedical research and application. Polycaprolactones can be synthesized by polymerization with anionic, cationic, and coordination catalysts. For the anionic catalytic system, tertiary amines, alkali metal alkoxides, and carboxylates are necessary for the polymerization. For the cationic

polymerization system, several catalysts, including protic acids, Lewis acids, acylating agents, and alkylating agents, are often used. The coordination polymerization system is used for synthesizing high-molecular-weight polymers. Catalysts for this type of polymerization include stannous octoate, alikoxides, and metallic elements such as Al, Sn, Mg, and Ti. Polycaprolactone materials usually have the following thermal properties: melting temperature ~60ºC and glass transition temperature ~−60ºC. Mechanically, polycaprolactone materials can be stretched to a strain about 0.3 (~30% elongation) at a yielding stress about 11 MPa. The elastic modulus is about 0.3 GPa.

Polycaprolactone materials and polycaprolactone hybrids with other materials, such as hydroxyapatite, poly-l-lactides, and silica, have been used in a number of biomedical applications, such as scaffolding and repairing soft and bone tissues (Fig.1). Polycaprolactone materials can be degraded by hydrolysis. *In vivo* tests have shown that it takes about 2–4 years to completely degrade a polycaprolactone implant. Copolymerization or blending with glycolides and/or lactides increases the rate of degradation. *In vivo* animal tests have shown that polycaprolactone materials exhibit low toxicity and do not significantly influence the function of host cells and tissues.

Poly (β-hydroxy butyrate)

Poly (β-hydroxy butyrate), or PHB, is a polymer of β-hydroxybutyrate. Poly (β-hydroxybutyrate) can be generated by natural fermentation of the bacterium *Alcaligenes* and can also be synthesized by the plant flax or linumusitatissimum, which is genetically transfected with gene constructs required for the synthesis of poly(β-hydroxy butyrate), or other types of plants such as *Arabidopsis thaliana* and *Brassica napus*. Poly (β-hydroxy butyrate) is an amorphous material with a glass transition temperature of about −40ºC and a melting temperature of ~160ºC. The failure strain (strain when a material is extended to the failure point) of poly(β-hydroxybutyrate) is about 0.15 or 15%. When poly (β-hydroxybutyrate) is blended with other types of polymers, such as hydroxyl hexanoate, the failure strain can be increased depending on the relative contents of the polymer components. The poly (β-hydroxybutyrate)-containing copolymer can be fabricated into various forms and used for constructing tissue replacements (Fig.2). The degradation of poly (β-hydroxybutyrate) is induced by hydrolysis.

In vitro tests have shown that high-molecular-weight poly (β-hydroxybutyrate) films can be completely degraded at 25ºC in freshwater within 3 weeks.
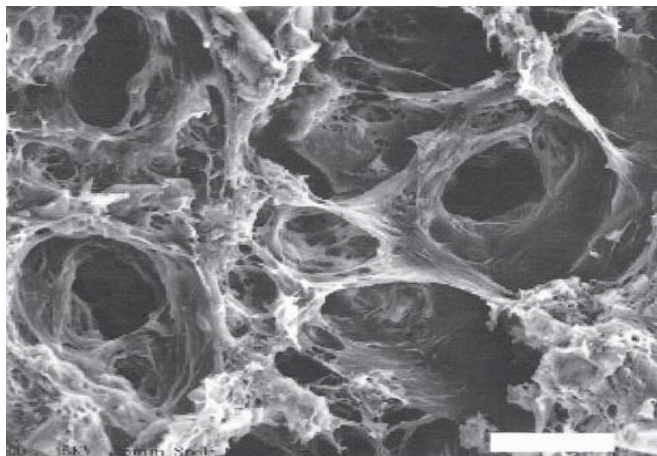
Figure 1. Scanning electron microscopic images of freeze fracture hydroxyapatite (HAP)/polycaprolactone (PCL) composite materials.

Polycarbonates

Polycarbonates are a group of polyesters, including poly (urethane carbonate), poly(ethylene carbonate), and poly(propylene carbonate). These polymers are synthesized with dihydroxy compounds and carbonyl chloride. For aliphatic poly (urethane carbonate), the glass transition temperature is about -18°C. The tensile failure stress of this polymer is about 50 MPa, and the failure strain is about 416%. Clearly, this is a highly extendable material. The degradation rate varies among different polycarbonate materials. For instance, poly(ethylene carbonate) tablets implanted in the rat can be degraded within about 21 days, poly(propylene carbonate) may last for 60 days, whereas poly (urethane carbonate) is stable for a much longer time. This class of polymeric materials are biocompatible and has been used for cell culture (Fig.3). These materials have also been used as biomaterials for constructing cardiovascular implants (intra aortic balloons, cardiac valves, vascular prostheses, pacemaker leads, ventricular assist devices and artificial heart diaphragms, heart valves, vascular grafts, and urethral catheters) as well as reconstructive implants (wound dressings, mammary prostheses, maxillofacial prostheses).

Polyamides

A *polyamide* is a polymer that is formed by joining monomers with an amide bond—CONH—. Natural proteins are amide-based polymers. Amino acids can be used to synthesize artificial polyamides. Typical examples are poly glutamic acid and poly lysine. Glutamic acid and lysine can also form copolymers with other types of amino acids. Unlike other synthetic polymers that have been tested in biomedical research, polymers based on amino acids are composed of naturally occurring molecules and thus possess low toxicity. Such a feature renders these polymers promising candidates as biomaterials for tissue repair and regeneration as well as drug delivery. Here, poly glutamic acid is used as an example to

demonstrate the principles of the synthesis, material properties, and potential biomedical application of polyamides.
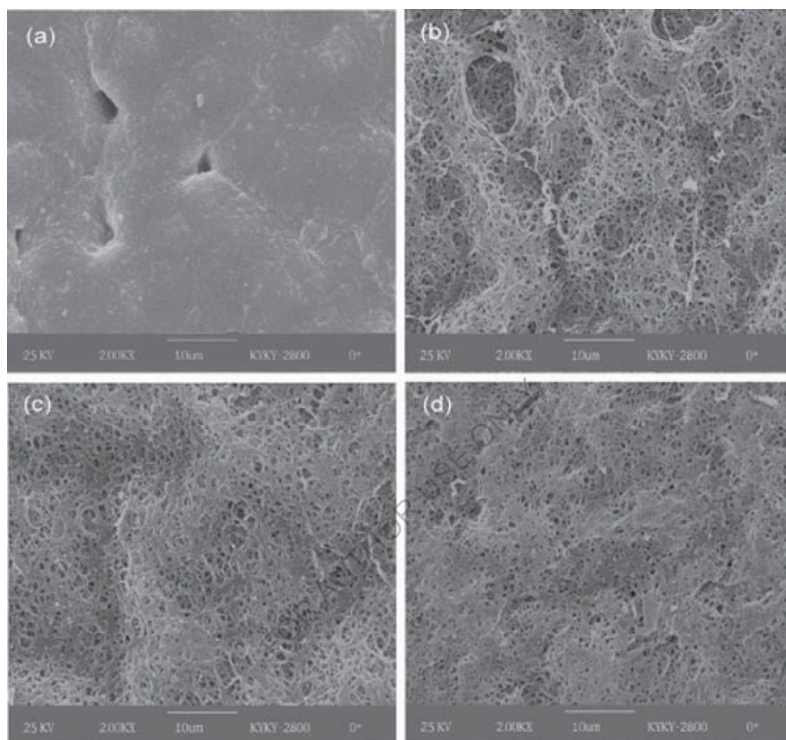
Figure 2. Scanning electron microscopic images of poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate)-based biomaterials:

(A) poly (3-hydroxybutyrate-*co*-3- hydroxyl hexanoate) materials;
(B) poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with 5% gelatin blend;
(C) poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with 10% gelatin blend;
(D) poly(3-hydroxybutyrate*co*-3-hydroxyhexanoate) with 30% gelatin blend.

Poly (glutamic acid) can be synthesized from poly (γ-benzyl-l-glutamate) by eliminating the benzyl group by using hydrogen bromide. Poly (glutamic acid) (PGA) can be degraded by enzymatic hydrolysis. In particular, cystein proteases play a critical role in the degradation of PGA. The time course of PGA degradation ranges from several hours to months, depending on the concentration of proteinase, temperature, and the composition of the polymer (with or without additional components). Poly (glutamic acid) exhibits little toxicity when implanted into an animal tissue. Animals can tolerate a single dose of ≤800 mg/kg and an accumulated dose of ≤1.8 g/kg. Polyglutamic acid exhibits little

immunogenicity in animal models. This type of materials can be used in various biomedical applications, such as tissue repair and regeneration as well as drug delivery.

Polyphosphazenes

Polyphosphazenes are inorganic biodegradable polymers that are constituted with a nitrogen–phosphorus (N==P) backbone. This type of polymer is synthesized via reactions of polydichlorophosphazene with amines or alkoxides in tetrahydrofuran or aromatic hydrocarbone solutions. Polymers with various side groups can be synthesized by mixing different components. The material properties of polyphosphazenes are dependent on the composition of the polymer. Biodegradable polyphosphazenes can be generated when amino acid derivatives are used as side groups. For instance, the ethylglycinato-derived polymers can be degraded into ammonia, phosphate, ethanol, and glycine. Experimental tests *in vitro* have demonstrated that amino acid-based polyphosphazenes can be degraded within several months. The rate of degradation is dependent on the type of amino acid selected for the sidegroups.
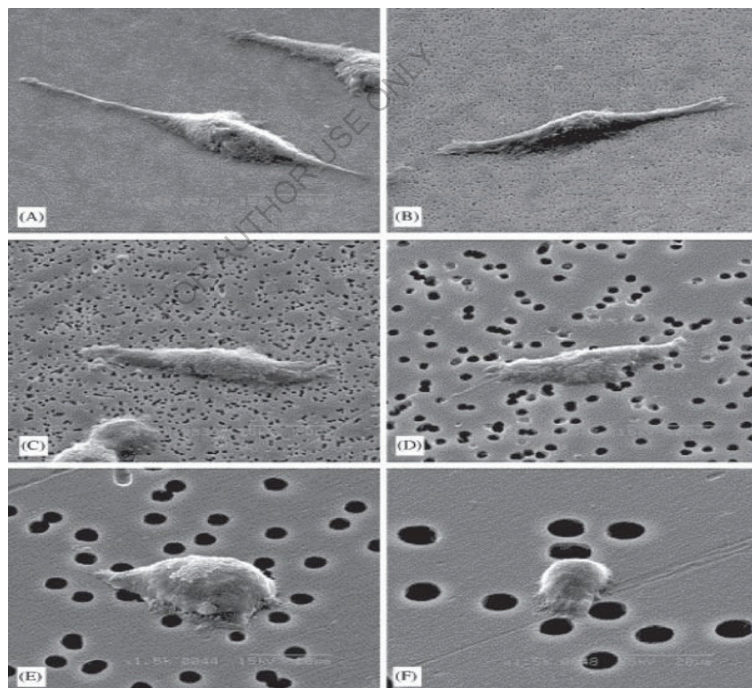
Figure 3. Scanning electron microscopic images of MG63 osteoblast-like cells (2-day culture) attached to the polycarbonate membrane surfaces with different micropore sizes: (A) 0.2, (B) 0.4, (C) 1.0, (D) 3.0, (E) 5.0, and (F) 8.0 μm in diameter of micropore sizes.

Polyphosphazenes have been used for constructing various structures, such as matrix scaffolds (Fig. 4), hydrogels, and microspheres, for drug delivery. The degradation of polyphosphazenes is sensitive to changes in temperature. Thus, the degradation rate of these polymers can be regulated by controlling environmental temperature. Such polymers can be used for constructing drug delivery carriers for temperature-related diseases. Other environmental factors, such as pH, may also influence the rate of degradation of polyphosphazenes. For instance, oxybenzoate-containing polyphosphazenes are pH-sensitive. The degradation of this type of polymer can be controlled by altering the content of oxybenzoate within a specifi ed range of pH. Such polymers can be used to deliver drug for diseases that result in a change in pH. *In vivo* animal tests with subcutaneous implantation have shown that polyphosphazenes materials exhibit low toxicity, induce little inflamatory reactions in host tissues, and are relatively safe for implantation and drug delivery.
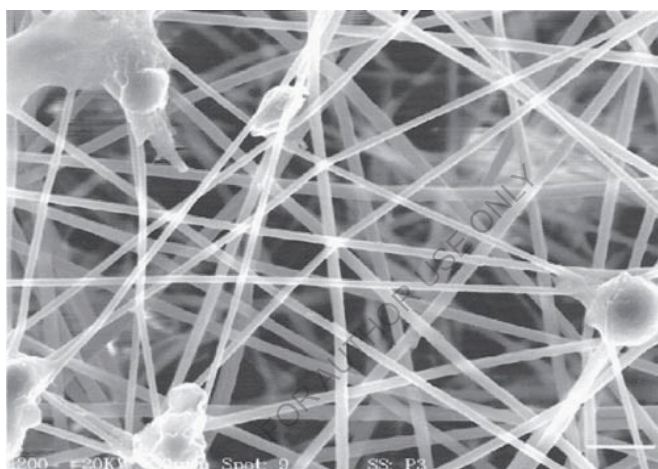
Figure 4. Scanning electron micrograph showing electrospun poly[bis(*p*-methylphenoxy) phosphazene fibre matrix with arterial endothelial cells after 24 h of culture.

Polyanhydrides

Polyanhydrides are polymers formed with anhydrides, compounds in which two carbonyl groups are joined with an oxygen atom, RCO—O—COR′, where R and R′ are any organic groups. The polymers are synthesized by reactions of diacids with anhydrides to form acetyl anhydride prepolymers. High-molecular-weight polyanhydrides can be formed from the prepolymers by melt condensation (180ºC for 90 min in vacuo). The addition of coordination catalysts, such as cadmium acetate and metal oxides, can facilitate the polymerization process, increasing the molecular weight of the polymer. Polyanhydrides can be dissolved in organic solvents such as chloroform and dichloromethane. Various components can be copolymerized with anhydrides to alter the solubility. Homopolymers of anhydrides usually exhibit a high level of crystallinity. Copolymerization with different components may reduce the crystallinity, producing more amorphous materials. Copolymerization also influences the mechanical properties of polyanhydrides.

Polyanhydrides are degraded by hydrolytic erosion. A number of factors influence the rate of polyanhydrides degradation. These include pH and copolymerization with different compounds. An increase in pH facilitates polyanhydrides degradation. The incorporation of different aliphatic monomers may facilitate the degradation of the polymer, whereas the addition of methylene groups into the polymer backbone reduces the rate of degradation. Thus, polyanhydrides with various levels of degradation can be synthesized by copolymerization with various compounds. Polyanhydrides can be used to construct various forms of matrix, such as disks and pellets, and can also be injected into target tissues. The injection of mixed polyanhydrides and therapeutic substances is a promising technique for controlled drug delivery. A number of studies have shown that polyanhydrides do not significantly influence the growth of cultured cells and exhibit little toxicity when implanted into target tissues in animal tests.

6. Biological materials

Collagen Matrix

Collagen matrix is found in mesenchymal and connective tissues, such as the subcutaneous tissue and bone, and the adventitia of tubular organs (blood vessels, airways, esophagus, stomach, and intestines). In mammalian tissues, there exist about 15 types of collagen matrix, namely, collagen types I–XV. Among these types of collagen, types I, II, III, IV, V, IX, XI, and XII are commonly found in connective tissues. Collagen types I, II, III, V, and XI are organized into filamentous structures, known as *collagen fibrils*, with a diameter of ~10–100 nm. These fibrils usually form large collagen bundles as found in subcutaneous tissues and the adventitia of tubular organs. Collagen types I and V are often found in the bone, skin, cornea, tendon, ligament, and internal organs such as the lung, liver, pancreas, and kidney. Collagen types II and XI are found in the cartilage, notochord, and intervertebral disks. Collagen type III is found in blood vessels, skin, and internal organs. Collagen types IX and XII are molecules that link other types of collagen fibril and are known as *fibril-associated collagens*. These types are found in the cartilage, tendons, and ligaments. In contrast to the filamentous collagen molecules, collagen type IV participates in the construction of a membrane-like structure, known as the *basal lamina*, which underlies epithelial and endothelial cells.

Collagen molecules play important roles in the constitution of mammalian tissues or organs. The collagen matrix serves as a structural framework that supports cells, helps organize cells into various forms of tissues and organs, and protects cells from mechanical injury. In addition, collagen matrix participates in the regulation of cellular activities such as cell survival, adhesion, proliferation, and migration. Collagen molecules can directly interact with cells via the cell membrane collagen receptors, or indirectly via the mediation of fibronectin, a matrix component that binds collagen molecules at one side and cell membrane matrix receptors, known as *integrins*, at the other side. The binding of collagen and fibronectin molecules to the integrin receptors initiate the activation of intracellular signaling pathways that stimulate or activate mitogenic processes, including cell survival, proliferation, and migration.

Given the structural and functional features, collagen matrix has long been used for constructing drug-delivery devices and scaffolds for tissue regeneration. Collagen matrix has been used in several forms: collagen gels, meshes, composites with different molecules, and decellularized natural matrix. Collagen gels and meshes are suitable for drug delivery,

whereas cell-free natural collagen matrix can be used as scaffolds or grafts for the repair or regeneration of various tissues and organs, such as blood vessels, airways, intestines, and bladder. Collagen gel can be spun into collagen fibres, which can be used to construct collagen scaffolds for tissue repair and regeneration (Fig. 5).

Native collagen matrix is a suitable material for the construction of tissue scaffolds. Such a material maintains the natural biological and mechanical characteristics and exhibits superior biocompatibility compared to *in vitro* crosslinked collagen gels or matrix. To prepare a native collagen matrix, mammalian tissue specimens can be collected from the submucosa of intestines, the adventitia of blood vessels, and the subcutaneous tissue. Cells in these specimens can be removed by various enzymatic and hydrolytic methods. Such treatments eliminate the cellular immunogenicity of allogenic tissues (note that extracellular matrix molecules exhibit neglible immunogenicity). The resulting cell-free collagen matrix can be tailored into a scaffold with a desired form and used for tissue repair or regeneration.
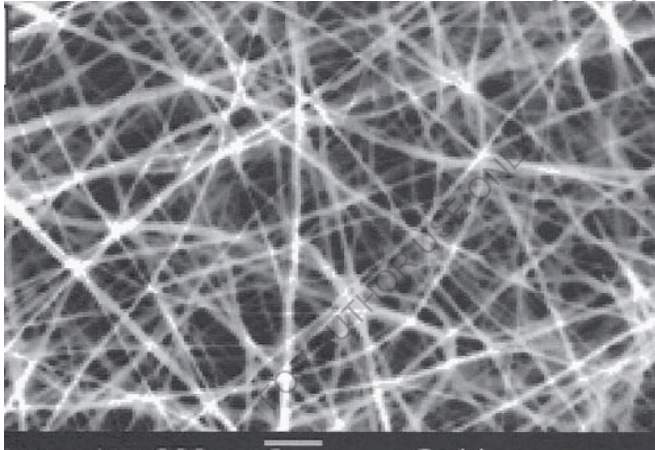
Figure 5. Scanning electron micrograph of electrospun collagen matrix.

Elastic Fibres and Laminae

Elastic fibres and laminae are major extracellular matrix components found in mesenchymal and connective tissues. Elastic fibres are present in the lung, connective tissue, the submucosa of intestines, and the wall of veins, whereas elastic laminae are found primarily in the media of large and medium arteries. Elastic fibres and laminae are composed of several proteins, including elastin, microfibrils, and microfibril-associated proteins. Elastin is the most abundant protein in elastic fibres and laminae. Mature elastin is a highly insoluble and hydrophobic protein, and is formed by crosslinking the 72-kDa elastin precursor, known as *tropoelastin*. Tropoelastin is produced by several cell types, including the smooth muscle cell, endothelial cell, and fibroblast, and is released into the extracellular space where crosslinking and elastin formation take place. Elastic fibres and laminae play an important role in the constitution of tissues and organs as well as in the maintenance of the stability of tissues and organs. For instance, multiple layers of elastic laminae are found in large arteries.

These laminae have long been known to contribute to the structural stability and mechanical strength of the arterial wall. Arteries are subject to extensive mechanical stress induced by arterial blood pressure. Without the support of the elastic laminae, vascular cells may be overstretched under arterial blood pressure. Elastic laminae also contribute to the elasticity of soft tissues, such as connective tissues and arteries. The recoil of the arterial wall is a critical mechanism for the continuation of blood flow during diastole when cardiac ejection is ceased. Elastic laminae have also been shown to serve as a signalling structure and play a role in regulating arterial morphogenesis and pathogenesis. An important contribution of elastic laminae is to confine smooth muscle cells to the arterial media by inhibiting smooth muscle cell proliferation and migration, thus preventing intimal hyperplasia under physiological conditions. In addition, elastic laminae exhibit anti inflammatory effects and inhibit leukocyte adhesion, activation, and transmigration relative to collagen matrix. These features render elastic laminae a potential material for vascular reconstruction. Furthermore, elastic laminae and elastin-containing structures can be used to prevent inflammatory reactions after surgery.

7. Polysaccharides

Polysaccharides are polymers composed of many monosaccharides bonded together by glycosidic bonds. There are a number of forms of natural polysaccharides, including glycogen, cellulose, alginate, chitosan, starch, and glycosaminoglycan. These polysaccharides are found in animals and plants, and play an important role for the survival and function of animals and plants. Glycogen is a polymer composed of glucose monomers and synthesized in animals for the storage of energy. Alginates are linear polysaccharides composed of β-mannuronic acid and α-guluronic acid, and are found in brown seaweed and in certain bacteria. Starch is a polymer found in plants and synthesized for the storage of energy. Cellulose is found in plants and bacteria. Chitosan is found in the shell of crabs and shrimps. One of the important properties of polysaccharides is their ability to form hydrogel. This property is the basis for polysaccharide-mediated drug delivery. Several types of polysaccharide, such as cellulose, chitosan, and starch, have been used as materials for tissue engineering and drug delivery.

Cellulose

Cellulose is a linear polysaccharide composed of d-glucose units jointed together by 1,4-β-glucosidic bonds. In plants, cellulose participates in the constitution of plant skeleton and cell wall. Cotton is a well-known cellulose-containing material. Cellulose molecules are often arranged in parallel, giving cellulose fibres high mechanical strength. Humans cannot use cellulose as an energy source because of the lack of β- glycosidase, which catalyzes the hydrolysis of β-glycosidic bonds (note that mammals have α-glycosidase that catalyzes the hydrolysis of glycogen and starch). Cellulose can also be produced by bacteria. Bacterial cellulose has been often used for tissue engineering and will be the focus here.

Several types of microorganism, including algae (*Vallonia*), fungi (*Saprolegnia*, *Dictystelium discoideum*), and bacteria (*Acetobacter*, *Achromobacter*, *Aerobacter*, *Agrobacterium*, *Pseudomonas*, *Rhizobium*, *Sarcina*, *Alcaligenes*, *Zoogloea*) can synthesize cellulose. Among these microorganisms, the bacterium Acetobacter xylinum, which is usually found in fruits, vegetables, and alcoholic beverages, has been used to generate cellulose for tissue engineering applications. In a culture medium, this bacterium can produce a network of cellulose fibres. The cellulose fibres can be collected, fabricated into desired forms, and used to construct scaffolds for tissue engineering applications. The bacterial

cellulose synthesized by *Acetobacter xylinum* is similar to the plant cellulose in molecular composition. Both types of cellulose contain d-glucose. However, bacterial cellulose exhibits a higher crystallinity, higher water absorption capacity or lower hydrophobicity, higher mechanical strength, and finer molecular arrangement compared to the plant cellulose. Cellulose and its derivatives, such as cellulose nitrate, cellulose acetate, and cellulose xanthate, can be easily fabricated into desired forms. Unlike other polysaccharides, such as glycogen and starch, cellulose exhibits low water solubility and, therefore, a low rate of degradation when implanted into an animal tissue. A decrease in the crystallinity and hydrophobicity of cellulose usually results in an increase in the biodegradability of cellulose. Given the chemical composition, bacterial cellulose is highly biocompatible and nontoxic to the host. Furthermore, bacterial cellulose is a highly mouldable material and can be used to fabricate scaffolds with desired forms. Cellulose-based materials have been used in a number of biomedical applications. These include construction of cellulose membranes for hemodialysis, construction of enzyme carriers for biosensors, drug delivery, construction of scaffolds for the regeneration of various tissue types, such as the bone, cartilage, liver, skin, and blood vessels. These investigations have consistently demonstrated that cellulose-based materials elicit little inflammatory and toxic reactions. Cellulose has been proven a promising material for the construction of tissue regenerating scaffolds.

Alginates

Alginates are linear polysaccharides composed of β-mannuronic acid and α-guluronic acid. Alginates are found in brown seaweed and in certain bacteria. The content of β-mannuronic acid and α-guluronic acid may vary depending on the plant or bacterial species from which alginates are obtained. Alginates can be used to form hydrogel and matrix. Divalent cations, such as Ca^{2+} and Mn^{2+}, can initiate alginate gelation by linking α-guluronic acid units between different polymer chains. The feature of gelation renders alginates a potential material for tissue engineering applications, such as cell seeding and transplantation, tissue repair, and drug delivery. Alginate gels with various mechanical properties can be generated under different gelling conditions and by using different crosslinkers. Numerous studies have been conducted to test the elastic and shearing mechanical properties. Under compressive forces, alginate matrices exhibit elastic modulus ranging from 1 to 1000 kPa, depending on gelling and experimental conditions. Similarly, the shear modulus of alginate matrices spreads widely from 0.02 to 40 kPa under different experimental conditions. Under tensile forces, the maximal tensile strength or failure stress of alginate gels ranges from 3 to 35 kPa and the maximal or failure strain is from 0.3 to 1.25, depending on the composition of alginates and the strain rate applied. Alginate gels cross-linked by Ca^{2+} have been used for a number of biomedical applications. One of the applications is alginate-mediated gene delivery. Alginate microspheres have been fabricated to carry genes of interest. The alginate microspheres can be delivered to target tissues, where the gene is released. Because of the biodegradability of alginates, genes can be released in a controlled manner with the releasing rate depending on the rate of alginate degradation. Similarly, an alginate-based gel or matrix can be used to mediate controlled protein and drug delivery. In addition, alginate-based materials have been fabricated and used to mediate wound healing. Alginates can form a thin layer of gel when crosslinked by Ca^{2+}. Such a gel layer can be used to cover skin wound to prevent the loss of body fluids and bacterial infection. Alginate-based materials can be used to construct various forms of matrix scaffolds for the repair or regeneration of various tissue types such as the cartilage, liver, and bone. Alginate materials have also been used to construct capsules for cell transplantation. Cells can be encapsulated within alginate capsules

and delivered to target tissues (Fig.6). The alginate capsules can partially protect the enclosed cells from inflammation-induced injury.

Chitosan

Chitosan is a linear polysaccharide composed of d-glucosamine units jointed by β-1,4-glycosidic bonds with randomly inserted *N*-acetylglucosamine units. Chitosan is a partially deacetylated derivative of chitin, which is a copolymer of randomly distributed *N*-acetylglucosamine and *N*-glucosamine units. A polymer molecule with more than 50% *N*-acetylglucosamine units is known as *chitin*, and that with more than 50% *N*-glucosamine units is called *chitosan*. Chitosan and chitin are found in the shell of crabs and shrimps and are similar to cellulose in structure. Chitosan and chitin can be collected from these shellfish sources. Chitosan is a semicrystalline molecule and is usually stable. It is insoluble in water, but soluble in acidic solutions (pH ∼5). The use of chitosan for tissue engineering relies partially on its gelation ability. Chitosan solutions can be gelled in methanol and under a high pH condition. A dried chitosan structure can be mechanically very strong. Chitosan molecules are usually positively charged and can bind to molecules with negative charges, such as glycosaminoglycans and alginates. A unique feature is that the charge density of chitosan is dependent on pH. Such a feature renders chitosan a candidate material for pH controlled drug delivery. When implanted *in vivo*, chitosan is degraded by lysozyme catalyzed hydrolysis. Chitosan is disintegrated into oligosaccharides. The rate of chitosan degradation is inversely proportional to the degree of crystallinity. The crystallization of chitosan is regulated by deacetylation. Chitosan molecules with increased deacetylation on the *N*-acetylglucosamine units exhibit increased crystallinity and reduced degradation. In a highly crystal form, it takes several months to degrade chitosan scaffolds *in vivo*. Amorphous chitosan exhibits more rapid degradation. Chitosan is a polysaccharide that can be fabricated into various forms of porous matrix. To produce a chitosan matrix, chitosan can be dissolved in acetic acid. The chitosan–acetic acid solution can be frozen and lyophilized to produce chitosan matrix. The freezing process induces the formation of ice crystals. The following lyophilizing process removes the ice crystals, allowing the formation of a porous matrix. The size of the pores can be controlled by altering the rate of ice crystal formation. Chitosan can be used to make materials with various mechanical properties. A pure chitosan material without apparent pores exhibits elastic modulus ranging from 5 to 7 MPa. However, the introduction of pores reduces the elastic modulus and mechanical strength. Porous chitosan materials could have an elastic modulus as low as 0.1 MPa. The failure strain or maximal strain of chitosan is also dependent on the porosity of the material. Nonporous chitosan materials can be stretched to a strain about 0.3, whereas a porous chitosan material can be stretched to a strain about 1. Porous chitosan exhibits a nonlinear mechanical behaviour, i.e., the mechanical behavior is dependent on the level of strain and stress. The material gains stiffness (with increased elastic modulus) when strain and stress are elevated.
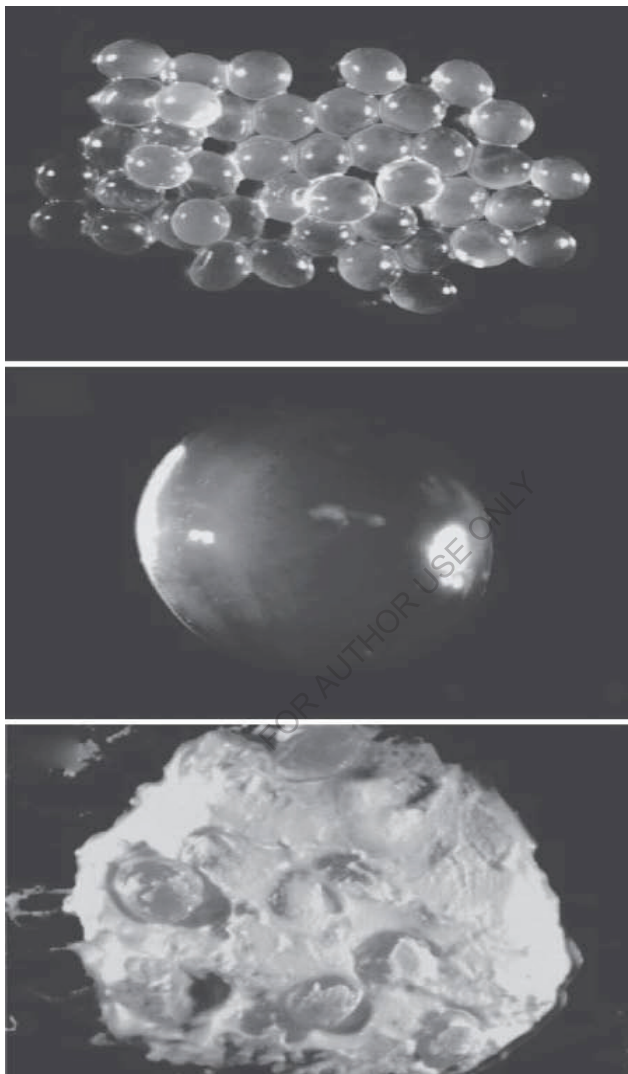
Figure 6. Cell-containing alginate beads for cell transplantation. Cells were encapsulated with alginate beads by dropping the cell–alginate mixture into an agitated bath of calcium chloride using a syringe. (A) A-low magnification image of the beads. (B) A higher-magnification image. (C) Cell–alginate beads were mixed into a calcium phosphate cement paste at a 54% volume fraction of alginate beads.

Chemical modifications significantly influence the mechanical properties of chitosan materials. For instance, the coating of a chitosan material with hyaluronic acid significantly increases the tensile strength of the chitosan material. This mechanical reinforcement is due to the formation of tight bonds between positively charged chitosan molecules and negatively charged hyaluronic acid molecules. Such reinforced chitosan materials are suitable for repairing tissues with high mechanical loads such as cartilage. Furthermore, the incorporation of hydroxyapatite or other calcium containing materials into chitosan or chitin can generate composite materials with increased mechanical strength. Such materials can be used for bone repair or regeneration. Given the molecular structure, mechanical properties, biocompatibility, and the capability of forming various matrix structures, chitosan and chitosan derivatives have been considered candidate materials for the engineering and regeneration of injured tissues and organs. Chitosan materials have been used to construct matrix scaffolds for seeding, culturing, and transplanting cells into target tissues. These materials have also been used as carriers for drug delivery. Several studies have shown that chitosan can serve as a gene transfer carrier. Genes mixed with chitosan-based materials have been successfully delivered to target cells in the knee joints in animal models. Chitosan-mediated gene transfer can also be carried out together with cell transplantation, enhancing therapeutic effects on target diseases.

Numerous investigations have shown consistently that chitosan and chitosan derivatives are relatively nontoxic and biocompatible. In particular, chitosan-based materials do not induce significant fibrous encapsulation around the implants. Although chitosan implantation induces leukocyte infiltration during the early period (within days), chronic inflammation does not occur significantly. The application of chitosan to cartilage repair and regeneration has demonstrated a beneficial effect on the recovery of injured cartilage tissues, such as stimulation of chondrocyte growth and expression of structural proteins. These observations have demonstrated the feasibility of using chitosan and chitosan derivatives as biomaterials for tissue regenerative engineering.

Starch

Starch is composed of d-glucose and is a form of polysaccharide for the storage of energy in plants. It can be found in all plant seeds and tubers. There are two forms of starch: amylase and amylopectin. Amylose is a linear polymer with the d-glucose units joined by the α1,4-glycosidic bonds, whereas amylopectin contains branching polymer chains, in which the d-glucose units are joined by the α1,4-glycosidic bonds in the linear portion and those at branching points are joined by the α1,6-glycosidic bonds.

Corn starch is usually used in biomedical research. Starch can be blended with chemical compounds such as ethylene vinyl alcohol and cellulose acetate to make matrices that can serve as engineering scaffolds or cell seeding/culture substrates. The fabricated matrix can be reinforced by mixing with hydroxyapatite to form a composite material. Polymer matrices of various forms can be prepared by injection molding. Starch and its composites have been used as substrates for cell culture and carriers for cell transplantation. Starch based materials do not significantly influence the growth and function of cultured cells. These materials have also been used *in vivo* for several biomedical applications, including drug delivery and tissue repair and regeneration. While starch may not be mechanically strong, the addition of reinforcement compounds may enhance the mechanical strength. As starch is composed of natural d-glucose, starch-based materials are usually

nontoxic and biocompatible. Such features render starch a promising material for tissue regenerative engineering.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear polysaccharides composed of repeated disaccharide units. Each unit contains an uronic acid and amino sugar molecule. According to the type of the disaccharide unit, GAGs can be classified into several groups, including chondroitin sulphate, hyaluronate, keratin sulphate, and heparin sulphate. A chondroitin sulphate molecule is composed of a glucuronic acid and an *N*-acetylgalactosamine unit with a SO_4 group on the 4 or 6 carbon positions. A hyaluronate molecule contains a glucuronic acid and an *N*-acetylglucosamine unit. A keratin sulphate molecule contains a galactose and an *N*-acetylglucosamine unit with a SO_4 on carbon position 6. A heparin sulphate molecule contains a d-glucuronic acid and an *N*-acetyl-dglucosamine unit.

Glycosaminoglycans are found in mammalian connective tissues, such as the subcutaneous tissue, cartilage, and blood vessels. These molecules attach to core proteins and form proteoglycans, major extracellular matrix molecules known as *ground substances*. Heparin sulphate is found on the surface of vascular endothelial cells and is similar in structure and function to heparin, which is a potent anticoagulant. Glycosaminoglycans are characterized by several general features, including the presence of a high density of negative charges, high hydrophilicity and water solubility, and low crystallinity. However, there are differences in material properties between various GAGs molecules. For instance, hyaluronate is a large molecule and has a high gel-forming capability. These molecules absorb a large amount of water, constituting a major part of the extracellular matrix. Because of the gel-forming capability, hyaluronate is often used as a media for drug-delivery or a material for tissue repair and regeneration. The composition of hyaluronate may be modified to construct materials with various properties. For example, partial esterification of the carboxyl groups of hyaluronate molecules reduces the water solubility of the polymer and increases its viscosity. Extensive esterification generates materials that form water-insoluble films or gels. Thus, hyaluronate gels with desired properties can be prepared by altering chemical compositions. Compared to hyaluronate, other types of GAGs exhibit poor gel-forming capability *in vitro*. These GAGs alone have not been used extensively as biomaterials. However, negatively charged GAGs can bind tightly to positively charged molecules, such as chitin and chitosan, and form composite polymeric materials. Such composite materials can be used to form gels with various material properties by altering the relative contents of GAG and/or chitosan. Glycosaminoglycans and glycosaminoglycan-based composite polymers have been used to construct hydrogels and matrices for various biomedical applications, such as drug delivery, cell seeding and transplantation, tissue repair, and tissue regeneration. Since GAGs are natural molecules, they are biocompatible and do not cause significant toxic and inflammatory reactions. These molecules can be degraded at different rates, depending on the compositions of the materials. For fully esterified hyaluronate membranes, the lifetime is several months. A reduction in esterification increases the rate of degradation.

8. Metallic materials as Biomaterials

Several types of metallic material have been used as biomaterials. These include iron (Fe), chromium (Cr), cobalt (Co), nickel (Ni), titanium (Ti), molybdenum (Mo), and tungsten (W). These materials have also been used to create alloys, providing favorable properties for

the fabrication and performance of biomaterials. Typical examples of alloys include Co–Cr and Ti alloys. In addition, stainless steels have been developed and used as biomaterials. Because of their superior strength, elasticity, and endurance, metallic materials are often used for the repair and replacement of bones and joints. For the past several decades, these alloys have been well accepted for their performance, biocompatibility, and stability.

Stainless Steels as Biomaterials

Steels are artificially modified forms of iron with various carbon contents and are characterized by mechanical hardness, elasticity, and strength. Thus, steels are considered candidate materials for the repair of the skeletal system. The mechanical features of steels are dependent on the content of carbon and temperature. The crystal structure of iron, which determines the mechanical characteristics of iron, can be modulated by altering the treatment temperature and carbon concentration. At a relatively cold temperature, say 20ºC, iron atoms are organized into a unit structure with a *body-centered cubic* form. In each unit, eight neighboring atoms are symmetrically localized to the corners of an imaginary cube with one atom at the cube center. An increase in temperature to a certain degree can induce a transformation of the atomic structure from the body-centered cubic form into a unit structure with a *face-centered cubic* form, in which the atoms are localized to the faces of an imaginary cube. Carbon atoms can be integrated more easily into the iron unit structure with the face-centered cubic form than that with the body-centered cubic form. Thus, an appropriate alteration in temperature facilitates the integration of carbon into the iron. Carbon integration enhances the stability, hardness, and strength of the iron. However, the solubility of carbon in iron is relatively low. An excessive level of carbon induces carbon precipitation, a problem influencing the endurance and mechanical properties of the steel. An appropriate concentration of carbon is about 0.03%. A common problem for using steels as biomaterials is corrosion. To resolve such a problem, chromium has been added to steels, rendering the steels stainless. For the manufacturing of biomaterials, chromium is used at a concentration ranging from 17 to 20%. However, the use of chromium introduces a problem: the mixing of chromium and carbon can form carbides, which enhances carbon precipitation. An approach used to mitigating carbide formation is to add nickel to the steel. Nickel can stabilize the iron structure, prevent carbide formation, and enhance corrosion resistance. Nickel is used at a concentration of 12–14% in steels as biomaterials. The chromium–nickel stainless steel has a high yielding strength (>170 MPa) and is considerably corrosion-resistant. However, corrosion can still occur when steel materials are implanted into the body. Thus, this material is often used to fabricate temporary implants such as fracture plates and nails.

Co–Cr Alloys as Biomaterials

Co–Cr alloys are metallic mixtures containing primarily Co and Cr as well as various amounts of other elements, such as Ni, Mo, Fe, C, Si, Mn, and Ti. There are two types of Co–Cr alloy that have been fabricated and used as biomaterials: CoCrMo and CoNiCrMo alloys. The CoCrMo alloys are composed of Co 63–68%, Cr 27–30%, and Mo 5–7%. The CoNiCrMo alloys consist of Co 31.5–39%, Ni 33–37%, Cr 19–21%, and Mo 9–10.5%. These alloys possess high yielding strength (~450 MPa for the CoCrMo alloys, and 240–655 MPa for the CoNiCrMo alloys). The CoCrMo alloys can be cast, while the CoNiCrMo alloys can be forged, into implants of desired shapes. Both types of alloy are highly corrosion-resistant. These alloys are suitable materials for the fabrication of artificial bones and joints.

Titanium and Titanium Alloys as Biomaterials

Titanium is a metallic material that is characterized by superior hardness, corrosion resistance, and lightness (4.5 g/cm3 compared to 7.9 g/cm3 for iron). Given such features, titanium has been used as a biomaterial for the replacement of bones and joints. Titanium materials usually contain several elements, such as nitrogen, carbon, hydrogen, oxygen, and iron. The contents of these elements are very low, with nitrogen ranging from 0.03–0.05%, carbon about 0.1%, hydrogen about 0.015%, oxygen 0.18–0.40%, and iron 0.2–0.5%. Titanium has been used to make alloys. A typical titanium alloy is Ti6Al4V, which contains ~6% aluminum, ~4% vanadium, ~90% titanium and low contents of nitrogen, carbon, hydrogen, oxygen, and iron. The addition of aluminum and vanadium increases the strength and corrosion resistance of the titanium alloy. For instance, pure titanium possesses yielding strength ranging from 170 to 485 MPa, whereas Ti6Al4V exhibits yielding strength ~795 MPa. Other types of titanium alloys have also been created to provide more features suitable for the performance of titanium alloys as biomaterials. Examples include Ti13V11Cr3Al and Ti13Nb13Zr. The Ti13V11Cr3Al alloy contains ~13% vanadium, ~11% chromium, ~3% aluminum, and ~73% titanium. The addition of these elements enhances the strength of the titanium alloy. The Ti13Nb13Zr alloy is composed of ~13% niobium, ~13% zirconium, and ~74% titanium. The addition of these elements enhances the corrosion resistance of the titanium alloy.

Potential Problems with Metallic Materials

There are two potential problems with the use metallic materials as biomaterials. These are corrosion and bioincompatibility. These problems potentially influence the performance and endurance of metallic biomaterials, especially when these materials are used to replace bones and joints that are subject considerably high mechanical loads. Corrosion is a process of metal degradation induced by chemical reactions, primarily oxidization. When subject to water-based solutions containing dissolved oxygen and ions such as chloride and hydroxide, metal atoms react with these species and form oxide or hydroxide compounds. These compounds detach from the metal surface and dissolve in the solution. The metal is degraded gradually. Since the physiological fluids in the human body contain oxidative chemical species, providing a harsh environment for metallic implants, corrosive degradation of metals occur at various rates, depending on the type of the metal and the local environment. Iron and steels can be corroded easily in the presence of water and oxygen, whereas chromium, nickel, and titanium are considerably corrosion-resistant. The concentration of oxygen in the interstitial fluids varies considerably in the different compartments of the body. Such variations significantly influence the rate of metal corrosion. An increase in oxygen concentration facilitates metal corrosion.

In addition to chemical factors, physical factors such as mechanical loads and friction accelerate metal corrosion. For instance, repetitive deformation of metal implants can induce mechanical fatigue, which facilitates chemical corrosion, a phenomenon known as *fatigue corrosion*. Shearing motions between two implants induces damage of the protective passivation layer, contributing to corrosion, which is known as fretting corrosion. These mechanical factors should be taken into account in the design of metallic implants.

There are several methods that can be used to measure the rate of corrosion. These include the estimation of the number of ions liberated from a metal per unit time, the measurement of the depth of the metal corroded away, and the measurement of the loss of the

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metal weight due to corrosion per unit time. These are fairly straightforward methods and can be applied to *in vitro* tests and *in vivo* tests in animal models.

Several approaches can be used to reduce the rate of metal corrosion. For steel-based implants, the addition of chromium can significantly reduce the rate of implant corrosion, since chromium can form a stable passive chromium oxide film on the steel surface. Modulation of carbon contents may also influence the rate of steel corrosion. Since excessive carbon content induces carbon precipitation, which may facilitate steel corrosion, lowering the carbon content is an effective approach to reduce the rate of steel corrosion.

Other metallic materials, such as cobalt and titanium, are considerably resistant to corrosion, since these metals are inert in physiological fluids and can form a passivating oxide film. Alloys based on these metallic materials exhibit improved resistance to corrosion. Although corrosion-resistant alloys are used, corrosion still occurs in artificial metallic bones and joints. Metallic corrosion often causes local swelling and pain, and influences the function of the artificial implants. Corrosion can be detected by x-ray examination.

At surgery, inflammatory reactions and metal debris can be found in tissue surrounding the metallic implants. Because corrosion accelerates metal wear and fatigue failure, metallic implants with severe corrosion should be replaced.

9. Ceramics as Biomaterials

Ceramics are a group of inorganic, polycrystalline, and refractory materials, including metallic oxides, carbides, silicates, hydrides, and sulphides. Ceramics are characterized by several physical properties, including the hardness, inertness to physiological ionic fluids, and resistance to high compressive stress. Given such properties, ceramics have been used as biomaterials for the replacement of bones and teeth. In terms of their interaction with biological tissues, ceramics can be classified into several types: bioactive, bioinert, and biodegradable ceramics. The characteristics and applications of these ceramics are discussed here.

Bioactive Ceramics

Bioactive ceramics are ceramics that can interact and form bonds with surrounding tissues. Such ceramics can be used as "adhesives" for prostheses, enhancing the attachment of prostheses to adjacent tissue. Given the mechanical strength and hardness, this type of ceramic is often used as adhesive for orthopedic applications such as the repair and replacement of bones and joints. A major type of bioactive ceramic is glass ceramics. This type of ceramics is constructed with SiO_2, CaO, Na_2O, and P_2O_5. The adhesive properties of glass ceramics are dependent on the formation of a surface layer composed of calcium phosphate and silicon oxide (SiO_2). Bioactive glass ceramics may not only serve as structural materials, but also play a role in regulating the function of host cells. For instance, silicon–calcium glass ceramics, once implanted into the skeletal system, can release silicon and calcium ions, which stimulate osteoblast growth and differentiation. Such a process involves genes that encode proteins responsible for the regulation of cell mitosis and differentiation. In addition, a controlled release of soluble calcium and silicon from a composite material composed of bioactive glass and resorbable polymer has been shown to enhance the generation of vascularised soft tissues. Thus, by controlling the compositions and releasing

rate of silicon and calcium, bioactive glass ceramics can be used to mediate the growth of bone tissues.

Bioactive glass ceramics have been used not only for bone replacement but also for soft tissue regeneration. Recent studies have shown that bioglass-coated polystyrene scaffolds stimulate the proliferation of cultured fibroblasts. Such an influence is dependent on the concentration of the coating bioactive ceramics. An excessive concentration of bioglass induces a reduction in the rate of cell proliferation, in association with a change in cell shape. A limited number of experiments have demonstrated that low concentration of bioglass (0.01%) may stimulate the expression and release of vascular endothelial growth factor. *In vivo* experiments have shown that bioglass-coated scaffolds can be well tolerated for up to 42 days when implanted subcutaneously in the rat. While fibroblasts actively adhere to poly(glycolic acid) (PGA) meshes, they rarely adhere to the bioglass particles. These observations demonstrate that bioactive glass ceramics can be used as compounds for the fabrication of composite scaffolds for soft tissue engineering.

Bioinert Ceramics

Bioinert ceramics, including alumina, zirconia, and carbons, have been used as biomaterials. These ceramics are generally corrosion-resistant and wear-resistant. They do not cause significant toxic, inflammatory, and allergic reactions and are relatively biocompatible. These ceramics possess common ceramic characteristics such as hardness, low friction, and resistance to compressive stress. Because of these characteristics, bioinert ceramics are often used to fabricate bone plates, screws, femoral heads, and middle ear ossicles. Alumina, or aluminum oxide (Al_2O_3), is a typical type of bioinert ceramics. Alumina exists in nature as crystal corundum. A crystal form of alumina can be synthesized by applying fine alumina powder to a flame of mixed oxygen and hydrogen. The mechanical strength of synthetic alumina is dependent on the grain size and porosity. Alumina with small grains and low porosity has high strength. A minimum of flexural strength 400 MPa and elastic modulus 380 GPa is required for using alumina as an orthopedic biomaterial. In general, alumina is a material that is characterized by hardness, low friction, inertness to physiological fluid environment, low toxicity, and low immunogenicity. These properties render alumina a suitable orthopedic biomaterial. Alumina has been used to fabricate artificial joints and total hip prostheses.

Biodegradable Ceramics

Biodegradable ceramics are ceramics that can be degraded and absorbed in a biological system. A number of biodegradable ceramics have been developed and used as biomaterials. These include calcium phosphate, aluminum calcium phosphate, coralline, zinc-calcium-phosphorous oxide, and zinc sulphate–calcium phosphate. Most biodegradable ceramics contain calcium. Biodegradable ceramics are often used for constructing artificial bones and drug delivery carriers, as well as to repair bone damages due to trauma, tumor removal, and pathological disorders. A biodegradable ceramic implant may serve as a temporary frame that guides the formation of the shape of remodeling tissues. The absorbed ceramic material can be replaced by growing tissue, eventually restoring the natural structure and function of the damaged tissue. Thus, biodegradable ceramics are suitable materials for orthopedic tissue regeneration.

Calcium phosphate is a typical biodegradable ceramic and has been used to fabricate artificial bones. Calcium phosphate can be crystallized into a form known as hydroxyapatite. Crystallized calcium phosphate can be very stiff and strong with an elastic modulus up to ~100 GPa. Note that the hardest tissue in our body, such as compact bones, dentin, and dental enamel, is composed of the crystal form of calcium phosphate with structure similar to hydroxyapatite. Thus, calcium phosphate is commonly used in orthopaedic regenerative engineering for the replacement and repair of malfunctioned bones. Calcium phosphate-based biomaterials are usually nontoxic and biocompatible. The biocompatibility of calcium phosphate-based bioceramics has been a topic of research in orthopedic regenerative engineering. Extensive investigations have shown that osteoblasts exhibit normal growth patterns when cultured on calcium phosphate materials. In addition, calcium phosphate biomaterials exert a stimulatory effect on the expression of osteogenic proteins and the proliferation of osteoblasts. These observations demonstrate the suitability of using calcium phosphate compounds as biomaterials for orthopaedic regenerative engineering.

Calcium phosphate ceramics can also be used to fabricate drug delivery devices. Drugs, hormones, or growth factors can be packed into biodegradable calcium phosphate ceramics for implantation and delivery into target tissues. With the degradation of the ceramic, drugs or proteins can be gradually released. By controlling the density or compounds of the drug delivery material, the rate of substance release can be regulated. Biodegradable ceramics can be mixed with biodegradable polymers, such as poly *d*,*l*,-lactic acidpolyethyleneglycol copolymer (PLA-PEG) to form composite materials. Such an approach enhances the capability of controlling the rate of substance release. A composite ceramic material can also serve as a scaffold for tissue regeneration. Biological active substances can be integrated into the scaffold for controlled substance release, which enhances the regeneration of injured tissues.

10. Success and Failures with Biomaterials and Medical Devices

Most biomaterials and medical devices perform satisfactorily, improving the quality of life for the recipient or saving lives. Still, manmade constructs are never perfect. Manufactured devices have a failure rate. Also, all humans differ in genetics, gender, body chemistries, living environment and physical activity. Furthermore, physicians also differ in their "talent" for implanting devices. The other side to the medical device success story is that there are problems, compromises and complications that occur with medical devices. Central issues for the biomaterials scientist, manufacturer, patient, physician and attorney are: (1) what represents good design; (2) who should be responsible when devices perform "with an *in*appropriate host response;" and (3) what are the cost/risk or cost/benefit ratios for the implant or therapy?

An example involving left ventricular assist devices (LVADs, sometimes incorrectly called artificial hearts) helps to clarify these issues. There are many complications with LVADs including clotting, strokes, blood damage and bacterial infection. A clinical trial called Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) led to following important statistics. Patients with an implanted Heartmate® LVAD (Thoratec Laboratories) had a 52% chance of surviving for one year, compared with a 25% survival rate for patients who took medication (the common therapy for congestive heart failure). Survival for two years in patients with the Heartmate® was 23% versus 8% in the medication group. Also, the LVAD enhanced the quality of life for the patients – they felt better, were less depressed, and were mobile. Note that patients

participating in the REMATCH trial were not eligible for a heart transplant. In the case of the LVAD, long-term clinical complications associated with imperfect performance of biomaterials does not preclude clinical success overall. The LVAD is better than the next best therapy.

These five characteristics of biomaterials science: multidisciplinary, multi-material, need-driven, substantial market and risk-benefit colour the field of biomaterials.

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Chapter 8
Tissue engineering

1 Introduction

Tissue engineering (TE) is the application of engineering principles to cell culture for the purpose of constructing functional anatomical units, normally for reconstructive surgery. There are three main components: cell biology, materials science (polymer/protein biochemistry), and biological engineering. TE aims to supply body parts for repair of damaged tissues and organs, without causing an immune response or infections, or using cadaveric tissue, or mutilating other parts of the patient. The dream is to blend the advantages of biological integration from living grafts with the ease, stability, and safety of prosthetic implants and as such it applies to most surgical specialities.

Two contrasting philosophies can be identified behind current approaches to tissue engineering. The first assumes that living cells possess an innate and self-sufficient potential for biological regeneration. The implication is that addition of suitable cell types to a suitable support matrix which allows proliferation and movement will result in an organized and functional tissue, resembling the tissue of origin. This is likely to be the simplest, most economic approach, where it can be applied. The second approach assumes that cells require a greater degree of control to produce new and functioning tissue structures. This approach reflects the understanding that, far from regenerating, mature tissues repair rather poorly *in vivo* and many cells do not organize in culture. This view leads to more complex solutions, generally supplying organizational cues to regulate resident cell function and provide spatial and synthetic control cues. Based on what is understood about tissue repair and regeneration biology it seems likely that epithelial and endothelial cell layers will prove better at organizing themselves through innate cell behaviours. In particular, cells such as keratinocytes and vascular endothelial cells make strong cell-cell interactions and form coherent sheets. Conversely, connective tissues or mesenchymal layers, in which extensive deposition and remodelling of collagen matrix is needed (central to many TE applications), tend not to form appropriate structures spontaneously.

The contribution of tissue repair processes is important in TE developments, because these are most surgical implants, including skin, blood vessels, heart valves, nerves, joint surfaces, and ligaments. Consequently, there is a spectrum of stages at which TE constructs can be designed to be implanted. At one end of this spectrum is the engineered tissue which is designed to guide, control, and improve the repair function, effectively acting as an implanted provisional tissue, for example to support peripheral nerve repair. At the other end of the spectrum is a fully functioning tissue ready to work in the patient. It is presently inconceivable that a functional peripheral nerve could be engineered to repair a defect or gap in a nerve tract. What is needed is a cellular repair tissue to guide and encourage regeneration of existing axons across the gap. This support function tends to be simpler to produce than a fully functioning implant, needing simpler and shorter *in vitro* tissue maturation periods, as this process occurs *in vivo*. However, tissue function will recover far more slowly and such constructs need to be functionally sophisticated to control local repair processes long after implantation.

Fully functioning implants include constructs designed to operate as large blood vessel implants or heart valves, where significant periods of patient recovery are impossible or impractical. Tissue applications falling between these two extremes can be tackled in a

mixture of ways. Current forms of TE skin, for example, tend to resemble repair or granulation tissue more than a skin graft, although graftable dermis would have many advantages.

2 Design stages for tissue engineering

A generalized scheme for the design of a TE construct is given in Table 1 with some specific examples. The surgical criteria are frequently based on previous procedures using grafts and prostheses, including ease of fixation, minimal patient discomfort, and the rapid restoration of function. The source of donor cells is critical to the design. However, this is often overshadowed by the question of whether autologous cells are essential. Where a mature implant is needed and little remodelling is expected, use of the patient's own cells is favoured. However, allogeneic cells become more attractive where the TE construct is designed as a temporary repair tissue. The implanted cells have a temporary role and are expected to be gradually lost in remodelling. Allogeneic and xenogeneic cells can be immunogenic and need special precautions to screen for infection. However, they are more amenable for use in the mass production of consistent, rapidly available, low cost constructs than is the case where the patient's own cells must be used. In this respect the use of cultured cells appears to provide a fortunate advantage, in that prolonged culture or cryopreservation seems to reduce the antigenicity of allogeneic cells. Early experiences with new commercially available TE skin (Apligraf™), using neonatal human foreskin cells, appear to support this view.

Design criteria related to the support material include their porosity and structure. The survival time of the material is important, as are its degradation products, cell adhesion characteristics, and ability to propagate surface guidance and mechanical cues. These features will, by accident or design, control the types of local cells which are recruited (macrophages, polymorphs, fibroblasts, smooth muscle cells, epithelial, or endothelial cells), their spatial organization, and the nature of the matrix they assemble. Three tissue examples are analysed (skin, urothelium, and peripheral nerve) in terms of their TE requirements and design features.

Table 1. Analysis of five major design stages useful to follow in TE constructions

Design stage	Demonstration examples Repair support Nerve repair Implant	Fully functioning Implant Large vessel Implant
List and rank the imperatives	Rapid, aligned axon regeneration Limited life of support material Ease of surgical fixation Non-fibrogenic	Immediately functional Mechanically strong (fluid pressure and suturing) Non-thrombogenic lumen Strong elastic wall
Select and optimize Materials	PLA/PGA synthetic polymer Collagen sponge Fibronectin fibrous tube (crosslinked)	Collagen gel or collagen-GAG Sponge PLA or PGA synthetic polymer
Select and isolate the cells	Schwann cells (SC) Perineural fibroblasts or crude cell extracts from nerve	Smooth muscle cells (SMCs) Endothelial cells (ECs) fibroblasts

Assembly and culture Stages	Seed SCs at staging points along material or seed SCs at distal end of construct Seed outer surface with fibroblasts Minimal/zero culture before implantation	Seed outer layers (SMCs) Seed lumenal surface (ECs) Long-term culture (under flow)
Incorporation of control and monitoring processes	Minimally inflammatory substrate Matrix Contact guidance substrate surfaces Material surface for rapid Migration Graded substrate degradation Slow release growth factors (e.g., NGF, NT3)	Fluid flow through lumen Growth factors (e.g., VEGF, FGF) Monitor for continuous endothelial layer Monitor for mechanical properties of wall Monitor for platelet adhesion to lumen

2.1 Tissue engineered skin

Tissue engineering of skin became feasible in 1975 with the demonstration that sheets of human keratinocytes could be grown in the laboratory in a suitable form for grafting. This was a simple, cohesive sheet of cells cultured from the donor on a feeder layer of fibroblasts. This technique has been extensively modified and applied clinically, but as a skin treatment without a dermal layer, its uses are limited. The epithelial component is able to regenerate in culture, because the cells grow as a continuous sheet over a suitable denuded surface, producing a continuous layer which progresses to form cornified layers. However, it is the underlying dermal layer which presents more difficulties, with its regular collagenous architecture, blood capillaries, nerves, and accessory organs such as sweat glands. There are various forms of implantable skin substitutes which can be considered as TE constructs. The first and simplest is a basic collagen-glycosaminoglycan sponge known as Integra™. Although Integra alone is a bioartificial material, rather than a TE construct, it has been used to cany seeded cells, as have a number of other collagen sponges and hyaluronan films. Integra consists of insoluble bovine collagen type I and the glycosaminoglycan chondroitin sulfate in a ratio of 98:2. Dermagraft™ consists of PGA polymer mesh of suitable pore size, seeded with human dermal fibroblasts from neonatal foreskins. As with Integra, this can be covered in a keratinocyte sheet at the time of implantation. Apligraf™ consists of human dermal fibroblasts seeded into a type I collagen gel and allowed to contract under tension. A layer of human keratinocytes is then seeded over the upper surface at the air-liquid interface. Both cell types in this construct are again derived from neonatal foreskins and so are allogeneic. Such TE constructs are available for surgical use, though with a limited shelf-life, presently in the order of five days. Clinical assessment of the performance and fate of Apligraf suggests that implants normally integrate well into surrounding tissues, forming a good skin cover. Importantly, there is no evidence of antibody formation to the bovine collagen, and little sign of rejection of the allogeneic cells in the construct, which are likely to disappear as the construct is remodelled.

2.2 Tissue engineered urothelium

As human urothelial cells and bladder smooth muscle cells can be cultured, it is likely that construction of tissue engineered urothelial implants will be possible. The criteria are that the final structures need to form elastic tubes or bladders able to remain patent (i.e., without strictures), and the implant should not allow crystal formation from urine or harbour local

infections. The structural requirements of the tissue are relatively simple in that an outer muscle layer should be lined on the lumenal surface by an intact, differentiating sheet of urothelium. Support materials tested have included resorbable polymers [poly(glycolic acid) and poly(lactic-co-glycolic acid) co-polymer: PGA and PLGA] and cross- linked collagen sponges. Isolated urothelial cells cultured on collagen sponges formed differentiated sheets of urothelium over the surface of the material, with minimal tendency to promote crystal deposition. Urothelial and bladder muscle cells seeded onto PGA scaffolds formed urothelium-like, vascularised bilayered tissues when implanted into rabbits. Recently, this technique has been applied to the tissue engineering of a functional bladder in dogs using a fibrous PGA polymer base, shaped into a bladder, and coated in PLGA 50:50 co-polymers. Muscle cells were seeded onto the outer surface of the bladder after which the lumenal surface was coated with pre-cultured urothelial cells, before implantation. Implanted bladders achieved near-normal performance and maintained this for up to 11 months.

2.3 Tissue engineered peripheral nerve implants
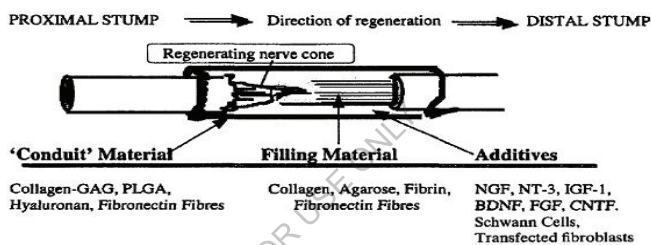
Figure 1. Scheme illustrating the basic design and some variations for peripheral nerve implants. Three elements are commonly used. The conduit outer layer (often the primary source of guidance). The filling material can also incorporate guidance elements but more often is for support of neural cells. Additives include a huge possible variety of growth and neurotrophic factors in many forms, combinations, ratios, and release modes.

Peripheral nerve injury is a common consequence of trauma and tumour resection surgery, with hundreds of thousands of reconstructive operations performed each year. Two criteria dominate approaches to assisted repair of peripheral nerve injuries. The first is that regeneration of axons should be guided as tightly as possible from their sprouting at the proximal stump to where they rejoin the degenerating distal stump on the far side of the defect. The second is that axonal regeneration across the nerve defect must be as fast as possible. Prolonged delay of reinnervation leads to irreversible muscle atrophy. Nerve guidance was achieved using silicone conduits attached between the nerve stumps and reported using tubes of bioresorbable materials such as PLGA co-polymer, collagen type I, and polymerized hyaluronan. The tube walls provide gross guidance, but no spatial cues are available to axons or Schwann cells away from the tube wall. Support for neurite outgrowth into the tube lumen has been provided by collagen and fibrin, with or without added growth factors, but again such gels provide minimal directional information.

Initial outgrowth for short distances from the proximal stump can be rapid, but for gaps of > 10-15 mm the process can slow or stop, probably due to a lack of supporting growth factors and Schwann cells. Continued growth can be achieved by adding purified growth factor or by seeding implants with Schwann cells. In another approach, optimization of the migration speed (as well as direction) has been described for orientated fibronectin implants,

by varying the proportion of fibrinogen in the polymer material, thus altering its surface adhesion properties.

2.4 Cardiac-Tissue Engineering

Heart disease remains the leading cause of death in developed countries. Tissue engineering offers a potential to grow *in vitro* functional equivalents of native myocardium for use in tissue repair and to investigate new ways to treat or prevent the disease. In this chapter, we focus on a tissue-engineering approach to the generation of a functional cardiac patch. In the first part, we review the clinical problem and the requirements for the tissue engineering of a functional cardiac patch in a form suitable for surgical implantation. In the second part, we give an overview of the representative most significant research in cardiac tissue engineering:
1. The biomimetic approach through the integrated use of cells, scaffolds, and bioreactors.
2. The mechanical stimulation of cardiomyocytes embedded in the collagen gels.
3. The cell-sheet approach.

We then describe in more detail the biomimetic approach to tissue engineering, where the tissue-engineering approach is designed to mimic the main factors present in the native myocardium: high cell density with multiple cell types, convective-diffusive oxygen transport through a capillary network, and excitation–contraction coupling. Finally, we discuss some of the current challenges and research needs.

2.4.1 Biometric approach to Cardiac tissue engineering

We describe here in detail the "biomimetic" approach we have developed for cardiac-tissue engineering. The approach involves the *in vitro* creation of immature but functional tissues by an integrated use of: (1) *differentiated cells*, (2) *biomaterial scaffolds* that serve as a structural and logistic template for tissue development and that biodegrade at a controlled rate, and (3) *bioreactors* that provide environmental conditions necessary for the cells to regenerate at a functional tissue.

In this approach, a bioreactor should ideally provide all necessary conditions in the *in vitro* environment for rapid and orderly tissue development by cells cultured on a scaffold. In general, a bioreactor is designed to perform one or more of the following functions: (1) establish a desired spatially uniform cell concentration within the scaffold during cell seeding, (2) maintain controlled conditions in culture medium (e.g., temperature, pH, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules), (3) facilitate mass transfer, and (4) provide physiologically relevant physical signals (e.g., interstitial fluid flow, shear, pressure, electrical stimuli) during cultivation of cell–polymer constructs.

We used the tissue-engineering model system to mimic the major factors present in the native myocardium. We focused on the following key parameters in the cell microenvironment: high cell density with multiple cell types, convectivediffusive oxygen transport through a capillary network, and orderly excitation contraction coupling (Table 2).

Table. 2: Factors governing cardiac-tissue development *in vitro* and *in vivo*

	In vivo	*In vitro*
Cells	High-density 5×10^8 cells/cm^3	$0.5–1 \times 10^8$ cells/cm^3
	Multiple cell types (myocytes, endothelial cells, fibroblasts)	Multiple cell types (myocytes, fi broblasts)
Oxygen and nutrient supply	Convection and diffusion	Convection and diffusion in perfusion bioreactor
Geometry	Capillary network (diameter 10 ìm, spacing 20 ìm)	Parallel channel array in the scaffold
Oxygen carrier	Hemoglobin	PFC emulsion (Oxygent®)
Excitation–contraction coupling	Electrical signal propagation Ventricle contraction	Electrical stimulation Construct contraction

2.4.2 Perfusion during Seeding Enabled Physiologic Cell Density

To provide an oxygen supply to the cells at levels necessary to maintain their viability, we developed a technique of seeding that involves (1) rapid cell inoculation into collagen sponges using Matrigel® as a cell delivery vehicle, and (2) transfer of inoculated scaffolds into perfused cartridges, with immediate establishment of the interstitial flow of the culture medium (Figure.2A). Forward–reverse flow was used for the initial period of 1.5–4.5 h to further increase the rate and spatial uniformity of cell seeding. Unidirectional flow of culture medium was maintained for the duration of cultivation. In this system, cells were "locked" into the scaffold during a short (10 min) gelation period and supplied with oxygen always during culture.

Cell distributions in the top, center, and bottom areas of a 0.65-μm-wide strip extending from one construct surface to the other are shown in Figure.2B. Constructs seeded in dishes had most cells located in the 100 μm thick layers at the top surface, and only a small number of cells penetrated the entire construct depth. Constructs seeded in perfusion had high and spatially uniform cell density throughout the perfused volume of the construct. Clearly, medium perfusion during seeding was a key for engineering thick constructs with high densities of viable cells, presumably due to enhanced transport of oxygen within the construct.
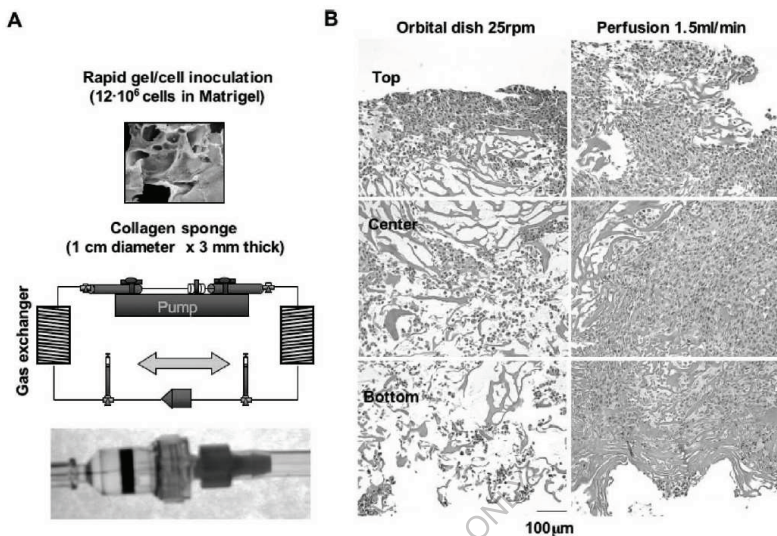
Figure.2 High-density perfusion seeding. (A) Cells are rapidly inoculated into collagen sponge using Matrigel and seeded, with alternating flow direction. (B) The method results in a spatially uniform distribution of cells at physiologically high concentrations and high cell viability. A full cross section through the center is shown for a construct inoculated with 12 million C2C12 cells and seeded for 4.5 h.

2.4.3 Perfusion During Cultivation Enabled Cell Viability and Aerobic Metabolism

Throughout the cultivation, the number of live cells in perfused constructs was significantly higher than in dish grown constructs, due to the perfusion of culture medium that was equilibrated in each pass with respect to oxygen and pH in an external-loop gas exchanger (Figure 3A). Notably, the final cell viability in perfused constructs cultured for eight days ($81.6 \pm 3.7\%$) was indistinguishable from the viability of the freshly isolated cells ($83.8 \pm 2.0\%$), and it was markedly higher than the cell viability in dish-grown constructs ($47.4 \pm 7.8\%$) (Figure 3B).

Consistently, the molar ratio of lactate produced to glucose consumed (L/G) was 1 for perfused constructs, indicating aerobic cell metabolism. In contrast, in orbitally mixed dishes, with convective flow of medium around the constructs and molecular diffusion within constructs, L/G increased progressively from 1 to 2, indicating a transition to anaerobic cell metabolism (Figure 3C). Cell damage, assessed by monitoring the activity of lactate dehydrogenase (LDH) in culture medium, was at all time points significantly lower in perfusion than in dish cultures. Perfused constructs and native ventricles had more cells in the S phase than in the G2/M phases, whereas the cells from dish-grown constructs appeared unable to complete the cell cycle and accumulated in the G2/M phase. Cells expressing cardiac-specific differentiation markers (sarcomeric α-actin, sarcomeric tropomyosin, cardiac troponin I) were present throughout the perfused constructs and only within approximately a 100-μm-thick surface layer in dish-grown constructs.

Spontaneous contractions were observed in some constructs early in culture and ceased after approximately five days of cultivation, indicating the maturation of engineered tissue. In response to electrical stimulation, perfused constructs contracted synchronously, had lower excitation thresholds, and recovered their baseline function levels following treatment with a gap junction blocker; dish-grown constructs exhibited arrhythmic contractile patterns and failed to recover their baseline levels. However, most cells were round and mononucleated, a situation likely due to the exposure of cardiac myocytes to hydrodynamic shear, in contrast to the native heart muscle, where blood is confined within the capillary bed and therefore not in direct contact with cardiac myocytes (Figure 3D). This motivated the design of scaffolds with arrays of channels that provide a separate compartment for medium flow.
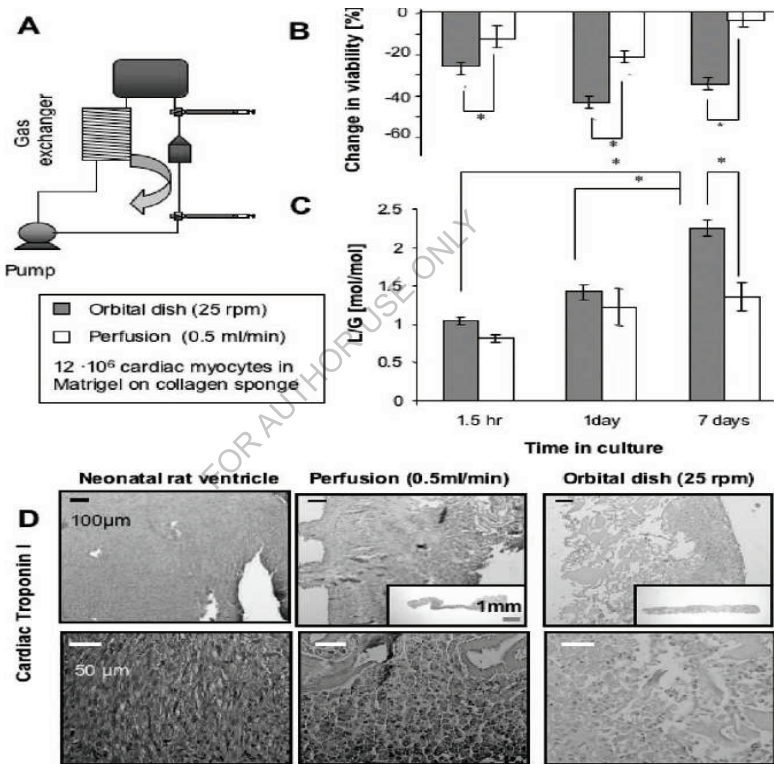
Figure 3. Convective-diffusive oxygen transport during culture maintains a high density of viable cells, aerobic cell metabolism, and uniform cell distribution. (A) Perfusion loop; (B) cell viability; (C) cell metabolism; (D) distribution of cardiac Troponin I–positive cells.

2.4.4 *In Vivo*–Like Oxygen Supply: Medium Perfusion, Channeled Scaffolds, and Oxygen Carriers

To test the feasibility of using channeled scaffolds, cardiac constructs were first engineered using a channelled collagen scaffold (UltrafoamTM, 1 cm in diameter × 3 mm thick) seeded with neonatal cardiac myocytes and cultivated in perfusion at 0.5 mL/min for 10 days. The channel maintained its initial diameter and was surrounded with a 300 μm thick tissue layer. However, collagen is not optimal for cardiac-tissue engineering due to its poor structural integrity. We thus explored the use of an elastomer, poly(glycerol sebacate), PGS pretreated with neonatal rat cardiofi broblasts for three days in orbitally mixed dishes, followed by the addition of rat cardiomyocytes and perfusion cultivation (Figure 4A). After only three days of culture, cells on scaffolds formed constructs that contracted synchronously in response to electrical stimulation (Figure 4C). The scaffold pores remained open, and the pressure drop measured across the construct was as low as 0.1 kPa/mm. To mimic the oxygen supply of hemoglobin, culture medium was supplemented by 10% v/v PFC emulsion [OxygentTM, kindly donated by Alliance Pharmaceuticals Corp. (San Diego, CA)]; constructs perfused with unsupplemented culture medium served as controls. Constructs were subjected to unidirectional medium flow at a flow rate of 0.1 mL/min provided by a multichannel peristaltic pump (IsmaTec). As the medium fl owed through the channel array, oxygen was depleted from the aqueous phase of the culture medium by diffusion into the construct space, where it was used for cell respiration. Depletion of oxygen in the aqueous phase acted as a driving force for the diffusion of dissolved oxygen from the PFC particles, thereby contributing to the maintenance of higher oxygen concentrations in the medium. Due to the small size of PFC particles, the passive diffusion of dissolved oxygen from the PFC phase into the aqueous phase was very fast, estimated not to be a rate limiting step in this system. For comparison, in unsupplemented culture medium, oxygen was depleted faster, because there is no oxygen carrier phase that acts as a reservoir (Figure 4B).

In PFC-supplemented medium, the decrease in the partial pressure of oxygen in the aqueous phase was only 50% of that in control medium (28 mmHg vs. 45 mmHg between the construct inlet and outlet at the flow rate of 0.1 mL/min). Consistently, constructs cultivated in the presence of PFC had higher amounts of DNA, troponin I, and Cx-43 and significantly better contractile properties than control constructs. In both groups, cells were present at the channel surfaces as well as within constructs. Improved construct properties were correlated with the enhanced supply of oxygen to the cells within constructs. To rationalize experimental data for oxygen transport and consumption in engineered cardiac constructs with an array of channels, we developed a mathematical model of oxygen distribution in cardiac constructs similar to the Krogh cylinder model. Concentration profiles of oxygen and cells within the constructs were obtained by numerical simulation of the diffusive-convective oxygen transport and its utilization by the cells. The model was used to evaluate the effects of the medium perfusion rate, oxygen carrier, and scaffold geometry on viable cell density. The model was used to define scaffold geometry and flow conditions necessary to cultivate cardiac constructs with clinically relevant thicknesses (5 mm). Oxygen profiles were modelled in a channel array consisting of channels 100 μm in diameter and 100 μm wall-to-wall spacing at physiologically high cell density $1 \cdot 10^8$ cells/cm^3. At 0.049 cm/s, the oxygen concentration increased significantly in both the tissue space and the channel lumen with the increase in circulating PFC emulsion, from 0% to 6.4%.

Although the oxygen concentration in the tissue space with physiological cell density increased considerably with the increase of circulating PFC concentration, from 0% to 6.4%,

we had to increase the flow rate, keeping the shear stress in the physiological range 1 dyn/cm^2, to provide enough oxygen for the entire 0.5-cm-thick construct. At our best conditions (0.135 cm/s and 6.4% PFC) (Figure 4D), oxygen is not depleted at any point within the tissue construct, and the minimum concentration of 33 μM is approximately five times above the *Km* (oxygen concentration at which the consumption rate in the tissue space is maintained at a maximum level).
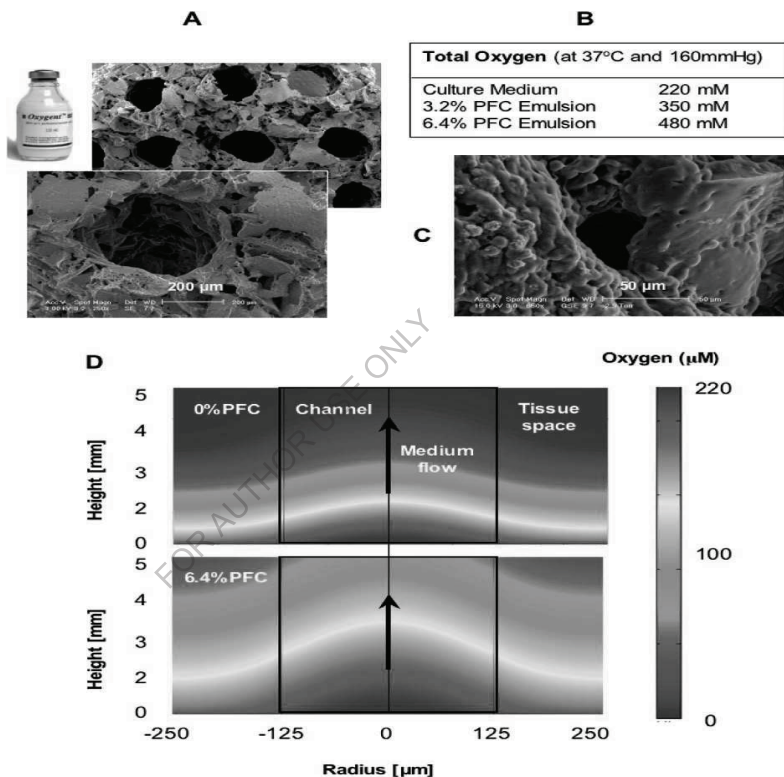
Figure 4. PFC emulsion increases the average oxygen concentration in the channel lumen and tissue space by increasing the oxygen-carrying capacity of the culture medium. (A) Channeled scaffolds were seeded with cells and perfused with culture medium that was supplemented with a perfl uorocarbon (PFC) emulsion. A scanning electron micrograph (SEM of a PGS scaffold with laser-bored channel array) is shown at two magnifications, before seeding with cells. (B) Supplementation of culture medium with PFC markedly increased the total oxygen content, as shown in table by data measured for two different concentrations of PFC. (C) SEM of cardiomyocytes cultured on channeled PGS scaffold perfused with PFC-supplemented culture medium. (D) Mathematical model of oxygen distribution in a perfused channeled scaffold with physiological cell density (10^8 cells/cm^3) in the tissue space. Data are shown for the superficial flow velocity of culture medium of 0.135

cm/s, for cultivations with unsupplemented culture medium (top image), and culture medium supplemented with 6.4% (vol/vol) of PFC (bottom image). Tissue constructs had channels that were 250 μm in diameter, spaced at 250 μm (wall-to-wall distance). Oxygen concentrations in the channel (outlined by a box in the center) and tissue space (on the sides) are shown by color code (on the right).

2.4.5 Excitation–Contraction Coupling: Electrical Stimulation

In native heart, mechanical stretch is induced by electrical signals, and the orderly coupling between electrical pacing signals and macroscopic contractions is crucial for the development and function of native myocardium (Severs, 2000). We therefore hypothesized that applying electrical signals designed to induce synchronous construct contractions would enhance cell differentiation and functional assembly of engineered tissue via physiologically relevant mechanisms. To test this hypothesis, cardiac constructs prepared by seeding collagen sponges ($6 \times 8 \times 1.5$ mm) with neonatal rat ventricular cells ($5 \cdot 10^6$) were stimulated using supra-threshold square biphasic pulses (2 ms duration, 1 Hz, 5 V). The stimulation was initiated after one to five days of scaffold seeding (three-day period was optimal) and applied for up to eight days.

The application of electrical stimulation during construct cultivation markedly enhanced the contractile behaviour. After eight days of culture, the amplitude of contractions was sevenfold higher in stimulated than in nonstimulated constructs (Figure 5A), a result of the progressive increase with the duration of culture. The excitation threshold (ET, the minimum voltage at which the entire construct was observed to beat) decreased (Figure 5B) and the maximum capture rate (MCR, the maximum pacing frequency for synchronous construct contractions) increased (Figure 5C) both with time and due to electrical stimulation, suggesting functional coupling of the cells. The shape, amplitude (100 mV), and duration (200 ms) of the electrical activity recorded for cells in constructs stimulated during culture were similar to action potentials reported for constructs that were mechanically stimulated during culture. After eight days, stimulated constructs demonstrated a remarkable level of ultra structural differentiation, comparable in several respects to that of native myocardium. Cells in stimulated constructs were aligned and elongated and contained centrally positioned elongated nuclei, in contrast to round cells in nonstimulated constructs, which had a high nucleus-to-cytoplasm ratio. Stimulated constructs and neonatal ventricles contained abundant mitochondria positioned between myofibrils, in contrast to nonstimulated constructs, containing mitochondria scattered around the cytoplasm and substantially larger amounts of glycogen.

Electrical stimulation induced the development of long, well-aligned registers of sarcomeres that closely resembled those in native myocardium (Figure 5D), representing a hallmark of maturing cardiomyocytes. The volume fraction of sarcomeres in stimulated eight-day constructs was indistinguishable from that measured for neonatal ventricles; in contrast, nonstimulated constructs contained only scattered and poorly organized sarcomeres. In stimulated constructs intercalated discs were positioned between aligned Z lines (Figure 5E) and were as frequent as in neonatal ventricles; gap junctions were also substantially better developed and more frequent.

Myofibers aligned in the direction of the electrical field lines, possibly in an attempt to decrease the apparent ET in response to pacing. In contrast, cells in nonstimulated constructs stayed round and expressed relatively low levels of cardiac markers. After eight days,

stimulated constructs exhibited a markedly higher density of Cx-43 than either early (three-day) or nonstimulated constructs. Notably, the improved contractile properties of electrically stimulated constructs were not reflected in any apparent differences in construct cellularity, cell damage, or cell metabolism, but correlated instead with cell differentiation.

Myofibers aligned in the direction of the electrical field lines, possibly in an attempt to decrease the apparent ET in response to pacing. Stimulated constructs and neonatal ventricles expressed high levels of cardiac Tn-I, sarcomeric α-actin, Cx-43, α-MHC, and β-MHC and contained elongated cells aligned in parallel (Figure 5F, G). In contrast, cells in nonstimulated constructs stayed round and expressed relatively low levels of cardiac markers. Cross striations characteristic of mature cardiac myocytes were detected in stimulated constructs and native ventricles but not in nonstimulated constructs.

In ongoing studies optical mapping to measure the impulse propagation in the constructs cultivated in the presence or absence of electrical field stimulation is being noticed. The constructs in the stimulated group had impulse propagation of 14.4 ± 2.7 V/cm, which was significantly higher than the impulse propagation in the nonstimulated group of 8.6 ± 3.0 V/cm. Our feasibility studies of implantation of the stimulated constructs in the rat model of myocardial infarction indicate that the constructs readily integrate with the host tissue and vascularize following implantation.
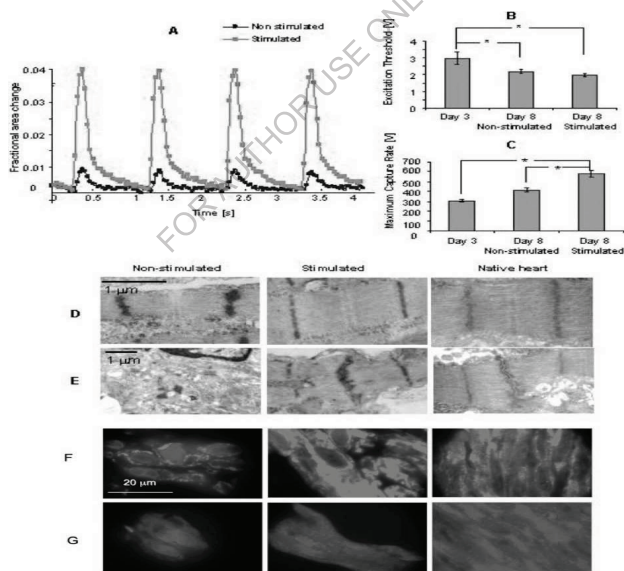
Figure 5. Effects of electrical stimulation on functional assembly of engineered cardiac constructs. (A) Contraction amplitude of constructs cultured for a total of eight days, shown as a fractional change in the construct size. Electrical stimulation increased the amplitude of contractions by a factor of 7. (B) The excitation threshold (ET) decreased and (C) the maximum capture rate (MCR) increased signifi cantly both with time in culture and due to

electrical stimulation. (*) denotes statistically signifi cant differences ($P < 0.05$; Tukey's post-hoc test with one-way ANOVA, $n = 5–10$ samples per group and time point). (D) The structure of sarcomeres and (E) gap junctions observed in micrographs of stimulated constructs after eight days of cultivation were remarkably similar to neonatal rat ventricles and markedly better developed than in control (nonstimulated) constructs. Representative sections of constructs stained for β-MHC (green, E) and α-MHC (red, G) cell nuclei are shown in blue.

2.4.6 Multiple Cell Types

Attempts to engineer cardiac tissue, including our previous studies, typically involve the use of cell populations enriched for cardiomyocytes (CM) by preplating and removing rapidly adhering cells. The presence of multiple cell types for the *in vitro* cultivation of heart tissue in their system, which involves the application of cyclic stretch.

Scaffold pretreatment with cardiac fibroblasts before the cultivation of cardiac myocytes can enhance functional assembly of the engineered cardiac constructs by creating an environment supportive of cardiomyocyte attachment, differentiation, and contractility. Neonatal rat heart cells were sorted and used to prepare three distinct cell populations: rapidly plating cells, identified as cardiac fibroblasts (CF); slowly plating cells, identified as cardiac myocytes (CM); and the unseparated initial population of cells (US). The structure and function of engineered constructs with respect to the use of the coculture (as compared to CM alone) and with respect to the regime of coculture (concurrent culture of CM and CF using US population; sequential coculture of CF and CM) formed with CF alone were used as an additional control. The cells were cultured for 11 days on porous scaffolds made of poly (glycerol sebacate) (PGS). Constructs in the CF + CM and CM groups exhibited similar fractions of myocytes (55%) and fibroblasts (20%) and similar amounts of cardiac-specific proteins. However, the constructs in the CF + CM group had significantly higher contractile amplitude than the CM group ($p < 0.05$) and significantly lower excitation threshold than the US group ($p < 0.05$). Also, the CF + CM group exhibited a stratified, 100- to 200-μm-thick tissuelike structure that contained some elongated CM, in contrast to relatively less organized cells in the US and CM groups. Thus, the sequential coculture of CF and CM on a synthetic elastomer scaffold created an environment supportive of cardiomyocyte attachment, differentiation, and contractile function. It is possible that CF played the role of a feeder layer for the cardiac cells and conditioned the scaffold by secreting collagen, which is a natural substrate for CM attachment. This approach can be used routinely to precondition the scaffolds for cardiac-tissue engineering.

2.5 Tissue engineered Liver

Cell-based therapies for liver failure offer the potential to augment or replace whole-organ transplantation. However, the development of such therapies poses unique challenges, stemming largely from the complexity of liver structure and function. The field of liver-tissue engineering encompasses several approaches collectively aimed at providing novel therapeutic options for liver disease patients as well as elucidating fundamental characteristics of liver biology. These approaches include the development of (1) *in vitro* model systems that recapitulate normal liver function, (2) extracorporeal bioartifical liver devices for the temporary support of liver failure patients, and (3) three dimensional implantable therapeutic constructs.

2.5.1 Cell sources for Liver Cell-based therapies

The choice of cell type is a critical parameter for any cell-based therapy. Table 3 highlights some of the key issues regarding the use of various cell sources. Immortalized hepatocyte cell lines, such as HepG2 (human hepatoblastoma), the HepG2-derived line C3A, HepLiu (SV40 immortalized) and immortalized fetal human hepatocytes have been utilized as readily available surrogates for hepatic tissue. However, it has been suggested that these cells lack the full functional capacity of primary adult hepatocytes, and for clinical applications there is a risk that oncogenic factors could be transmitted to the patient. Thus, the generation of conditionally immortalized lines and the incorporation of inducible suicide genes have been considered as potential precautionary measures. The use of primary hepatocyte-based systems could potentially eliminate these issues and provide the appropriate collection of liver functions. Primary porcine hepatocytes have been utilized in a range of BAL device configurations, with some encouraging results. However, the utility of xenogeneic porcine cells for human liver therapies is restricted by immunogenicity and the potential for xenozoonotic transmission of infectious agents such as porcine endogenous retrovirus (PERV).

Table 3: Cell sources for liver therapies

Cell source	Critical issues
Primary hepatocytes	Sourcing, expansion, phenotypic instability, immunogenicity, safety (xenozoonotic)
Human, xenogeneic	
Immortalized hepatocyte lines	Range of functions, genomic instability, safety (tumorigenicity)
Tumor-derived, SV40, telomerase, spontaneously immortalized	
Stem cells	Sourcing, differentiation effi ciency, phenotypic instability, immunogenicity, safety (tumorigenicity)
Embryonic, liver progenitors (hepatoblasts, oval cells), other lineages (HSC, MAPC)	

Primary human hepatocytes are ultimately the preferred cell type for cell-based therapies, and the development of primary hepatocyte–based approaches is the focus of substantial ongoing research. Yet progress has been hindered by the limited supply of primary human hepatocytes and certain aspects of hepatocyte physiology. Discussed in more detail later, hepatocytes exhibit a loss of liver-specific functions under many conditions *in vitro*. In addition, particularly for human hepatocytes, despite the significant proliferative capacity during regenerative responses *in vivo*, mature hepatocyte proliferation in culture is limited. As a result, alternative cell sources for liver cell–based therapies are being investigated, such as diverse stem cell populations, which can retain significant proliferative ability *in vitro* and exhibit either pluripotency or multipotency, thereby constituting a possible source of hepatocytes as well as other liver cell types. Preliminary evidence suggests that pluripotent embryonic stem cells can be induced to differentiate toward the hepatocyte lineage in culture and methods to improve the range of acquired hepatocyte functions as well as differentiation efficiency continue to be explored. Recently, an extracellular matrix microarray platform was utilized to examine the influence of combinatorial mixtures of matrix molecules on embryonic stem cell differentiation toward an early hepatic fate (Figure 6). In this system, an approximately 140-fold difference in the induction of an early hepatic marker (Ankrd17) was observed between the least and the most efficient conditions,

underscoring the importance of high-throughput technologies in the elucidation of factors affecting stem cell differentiation.
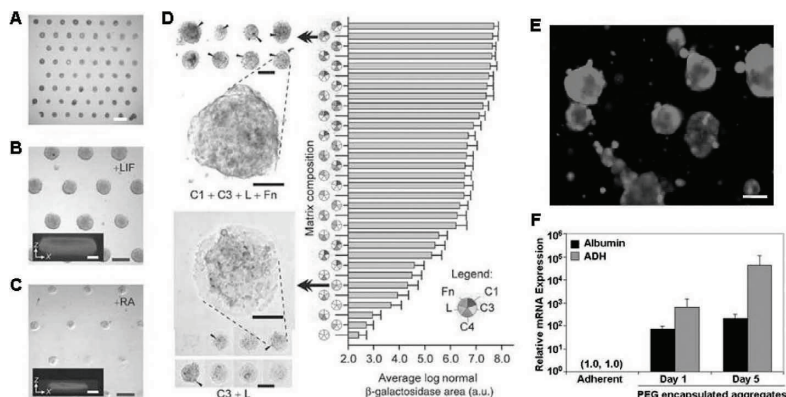
Figure 6. Platforms for studying hepatocyte differentiation of embryonic stem cells and liver progenitors. (A) Bright-fi eld alkaline phosphatase staining of day 1 ES cultures on ECM microarrays in 15% serum medium (scale bar, 1 mm). (B, C) Phase-contrast images of day 3 arrays cultured with LIF (B) and with RA (C). Cells cultured with LIF showed three-dimensional features (in B, inset x-z confocal section, 77-μm thickness). In contrast, RA-induced cells grew as a relatively thin sheet (in C, inset x-z section, 25-μm thickness). Scale bars, 250 μm (inset scale bars, 50 μm). (D) Bright-fi eld micrograph of selected X-gal-stained ECM microarray conditions after 3 d of culture in RA. C1 + C3 + L + Fn (top left images) induced higher *Ankrd17* reporter activity (arrowheads) than was seen in cells cultured on C3 + L (bottom left images). Scale bars, 250 μm. Magnified views of reporter activity: scale bars, 50 μm.
Bar graph: hierarchical depiction of "blue" image area (pooled data from four microarrays) for each of the matrix mixtures. Error bars, s.e.m. (n = 32). The C1 + C3 + L + Fn culture condition induced ~27-fold more β-galactosidase image area than the C3 + L cultures (Flaim *et al.*, 2005). (E) BMEL cell aggregates encapsulated within PEG hydrogels exhibit high viability. Fluorescent labeling distinguished viable (green) from nonviable (red) cells. Scale bar, 100 μm. (F) Expression of albumin (black bars) and alcohol dehydrogenase (ADH, grey bars) mRNA was determined by real-time quantitative RT-PCR at day 1 and day 5 following encapsulation of aggregated BMEL cells. Expression for each gene is displayed relative to basal expression exhibited by adherent BMEL cells before aggregation. The housekeeping gene, HPRT, was utilized as a normalization control, and data are presented as the mean ± SD (n = 3, independent experiments).

In addition to embryonic stem cells, various fetal or adult stem/progenitor populations have similarly been investigated. For example, multipotent adult progenitor cells (MAPC) derived from bone marrow have been shown to exhibit hepatocyte differentiation potential. Fetal hepatoblasts and oval cells are also intriguing possible cell types for liver cell–based therapies. Hepatic development proceeds through the differentiation of liver precursor cells, termed hepatoblasts, which exhibit bipotential differentiation capacity, defined by the ability

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to differentiate into both hepatocytes and bile duct epithelial cells (cholangiocytes). Notably, in certain types of severe and chronic liver injury, an adult progenitor cell population, termed oval cells, which shares many phenotypic markers and functional properties with fetal hepatoblasts, mediates compensatory liver repair through a similar differentiation program. Exposure to toxins or carcinogens that block hepatocyte proliferation, and consequently normal liver regeneration, results in the proliferation of oval cells within the liver and subsequent liver repair due to the differentiation of these cells. Recent work by Weiss and colleagues demonstrates the development of bipotential mouse embryonic liver (BMEL) cell lines that are derived from mouse E14 embryos and exhibit characteristics reminiscent of fetal hepatoblasts and oval cells. In particular, these cell lines are nontransformed and proliferative, demonstrate up-regulation of hepatocyte or bile duct epithelial markers under distinct culture conditions *in vitro* and exhibit the capacity to home to the liver and undergo bipotential differentiation *in vivo* within a regeneration context. This system highlights the potential of progenitor cell lines for tissue repair. As such, toward the eventual incorporation of BMEL cells into implantable tissue-engineered constructs, and discussed in more detail later in this chapter, BMEL cells have been successfully encapsulated within a biomaterial scaffold (PEG hydrogel), and their differentiation toward the hepatocyte lineage was demonstrated to proceed efficiently in this system.

Although diverse stem and progenitor cell populations exhibit vast potential regarding integration into hepatic therapies, many challenges remain, including the ability to dictate and enhance differentiation, particularly within multicellular systems. Furthermore, regardless of the cell source, the stabilization of hepatocyte functions remains a primary issue. Microenvironmental signals, including soluble mediators, cell-extracellular matrix interactions, and cell-cell interactions, have been implicated in the regulation of hepatocyte function. Accordingly, the development of robust hepatocyte *in vitro* culture models that allow for the controlled reconstitution of these environmental factors is a fundamental prerequisite toward a thorough understanding of mechanisms regulating hepatocyte processes and the improved functionality of liver cell-based therapies.

2.5.2 *In Vitro* Hepatic Culture models

An extensive range of liver model systems have been developed, some of which include: perfused whole-organs and wedge biopsies; precision-cut liver slices; isolated primary hepatocytes in suspension or cultured on extracellular matrix; immortalized liver cell lines; isolated organelles, membranes, or enzymes; and recombinant systems expressing specific drug metabolism enzymes. While perfused whole organs, wedge biopsies, and liver slices maintain many aspects of the normal *in vivo* microenvironment and architecture, they typically suffer from short-term viability (<24 h) and limited nutrient/oxygen diffusion to inner cell layers. Purified liver fractions and single-enzyme systems are routinely used in high throughput systems to identify enzymes involved in the metabolism of new pharmaceutical compounds, although they lack the complete spectrum of gene expression and cellular machinery required for liver-specific functions. In addition, cell lines derived from hepatoblastomas or from immortalization of primary hepatocytes are finding limited use as reproducible, inexpensive models of hepatic tissue.

However, such lines are plagued by abnormal levels and a repertoire of hepatic functions, perhaps most notably the divergence of nuclear receptor–mediated regulation of cytochrome P450 enzymes. Though each of these models has found utility for focused questions in drug metabolism research, isolated primary hepatocytes are generally considered

to be most suitable for constructing *in vitro* platforms for a multitude of applications. A major limitation in the use of primary hepatocytes is that they are notoriously difficult to maintain in culture, due to the precipitous decline in viability and liver-specific functions on isolation from the liver. Accordingly, substantial research has been conducted since the mid-1980s toward elucidating the specifi c molecular stimuli that can maintain phenotypic functions in hepatocytes. In subsequent sections, we present examples of strategies that have been used to improve the survival and liver-specifi c functions of primary hepatocytes in culture.

2.6 Tissue engineering of urologic structures

Tissue-engineering techniques are currently being investigated for the replacement of lost or deficient genitourinary structures, including urethra, bladder, male and female genital tissues, ureter and renal structures.

2.6.1 Urethra

Various strategies have been proposed to regenerate urethral tissue. Woven meshes of PGA, without cells, have been used to reconstruct urethras in dogs. PGA has been used as a cell transplantation vehicle to engineer tubular urothelium *in vivo*. Small intestinal submucosa (SIS) without cells was used as an on lay patch graft for urethroplasty in rabbits and a homologous free graft of acellular urethral matrix was also used in a rabbit model. Bladder-derived acellular collagen matrix has proven to be a suitable graft for repairing urethral defects in rabbits. The created neourethras demonstrated a normal urothelial luminal lining and organized muscle bundles. Results were confi rmed clinically in a series of patients with a history of failed hypospadias reconstruction whose urethral defects were repaired with human bladder acellular collagen matrices (Figure 7). An advantage of this material over nongenital tissue grafts for urethroplasty is that it is "off the shelf," eliminating the need for additional surgical procedures for graft harvesting and decreasing operative time and potential morbidity from the harvest procedure.
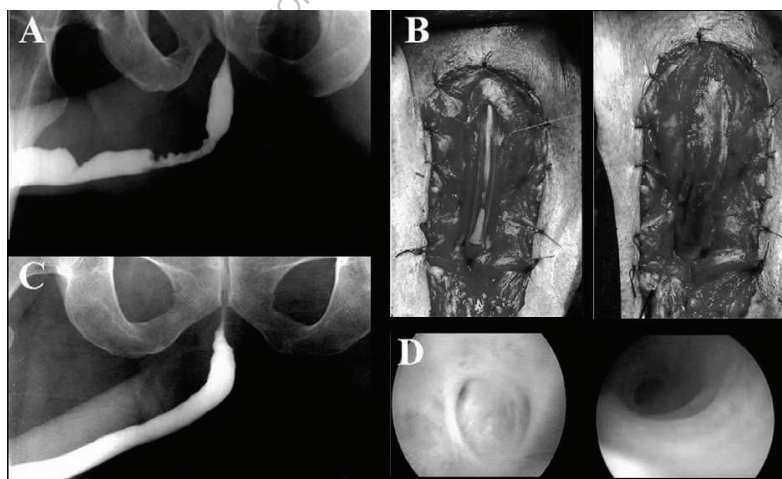
Figure 7. Tissue-engineered urethra using a collagen matrix. (A) Representative case of a patient with a bulbar stricture. (B) Urethral repair. Strictured tissue is excised, preserving the urethral plate on the left side, and matrix is anastomosed to the urethral plate in an onlay fashion on the right. (C) Urethrogram six months after repair. (D) Cystoscopic view of urethra before surgery on the left side and four months after repair on the right side.

The foregoing techniques, using non-seeded acellular matrices, were successfully applied experimentally and clinically for on lay urethral repairs. However, when tubularized repairs were attempted experimentally, adequate urethral tissue regeneration was not achieved, and complications, such as graft contracture and stricture formation, ensued (le Roux, 2005). Seeded tubularized collagen matrices have performed better than their non seeded counterparts in animal studies. In a rabbit model, entire urethral segments were resected, and urethroplasties were performed with tubularized collagen matrices, either seeded or nonseeded. The tubularized collagen matrices seeded with autologous cells formed new tissue, which was histologically similar to native urethra. Those without cells led to poor tissue development, fibrosis, and stricture formation.

2.6.2 Bladder

Gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, these tissues are designed to absorb specific solutes, and when they come in contact with the urinary tract, multiple complications may ensue, including infection, metabolic disturbances, urolithiasis, perforation, increased mucus production, and malignancy. Because of these problems, investigators have attempted alternative reconstructive procedures for bladder replacement or repair, such as the use of tissue expansion, seromuscular grafts, matrices for tissue regeneration, and tissue engineering with cell transplantation.

1. Tissue Expansion

A system of progressive dilation for ureters and bladders has been proposed as a method of bladder augmentation but has not yet been attempted clinically. Augmentation cystoplasty performed with the dilated ureteral segment in animals has resulted in increased bladder capacity ranging from 190% to 380%. A system progressively to expand native bladder tissue has also been used for augmenting bladder volumes in animals. Within 30 days after progressive dilation, neoreservoir volume was expanded at least 10-fold. Urodynamic studies showed normal compliance in all animals, and microscopic examination of the expanded neoreservoir tissue confirmed a normal histology. A series of immunocytochemical studies demonstrated that the dilated bladder tissue maintained normal phenotypic characteristics.

2. Seromuscular Grafts

Seromuscular grafts and deepithelialized bowel segments, either alone or over a native urothelium, have also been attempted. Keeping the urothelium intact avoids complications associated with the use of bowel in continuity with the urinary tract. An example of this strategy is to combine the techniques of autoaugmentation and enterocystoplasty. An autoaugmentation is performed, and the diverticulum is covered with a demucosalized gastric or intestinal segment.

3. Matrices

Nonseeded allogeneic acellular bladder matrices have served as scaffolds for the ingrowth of host bladder wall components. The matrices are prepared by mechanically and chemically removing all cellular components from bladder tissue. The matrices serve as vehicles for partial bladder regeneration, and relevant antigenicity is not evident. For example, SIS (a biodegradable, acellular, xenogeneic collagen-based tissue matrix graft) was first used in the early 1980s as an acellular matrix for tissue replacement in the vascular field. It has been shown to promote regeneration of a variety of host tissues, including blood vessels and ligaments. Animal studies have shown that the nonseeded SIS matrix used for bladder augmentation can regenerate *in vivo*.

In multiple studies using various materials as nonseeded grafts for cystoplasty, the urothelial layer regenerated normally, but the muscle layer, although present, was not fully developed. Often the grafts contracted to 60–70% of their original sizes, with little increase in bladder capacity or compliance. Studies involving acellular matrices that may provide the necessary environment to promote cell migration, growth, and differentiation are being conducted. With continued research, these matrices may have a clinical role in bladder replacement in the future. Recently, bladder regeneration has been shown to be more reliable using SIS derived from the distal ileum.

4. Cell Transplantation

Cell-seeded allogeneic acellular bladder matrices have been used for bladder augmentation in dogs. Trigonesparing cystectomy was performed in dogs randomly assigned to one of three groups. One group underwent closure of the trigone without a reconstructive procedure; another underwent reconstruction with a nonseeded bladder-shaped biodegradable scaffold; and the last underwent reconstruction using a bladder-shaped biodegradable scaffold that delivered seeded autologous urothelial cells and smooth muscle cells.

The cystectomy-only and nonseeded controls maintained average bladder capacities of 22% and 46% of preoperative values, respectively, compared with 95% in the cell-seeded tissue-engineered bladder replacements (Figure 8). The subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42% total compliance). The compliance of the cell-seeded tissue-engineered bladders showed almost no difference from preoperative values, which were measured when the native bladder was present (106%). Histologically, the nonseeded scaffold bladders presented a pattern of normal urothelial cells with a thickened fibrotic submucosa and a thin layer of muscle fi bers (Figure 8, middle parcel). The retrieved tissue-engineered bladders showed a normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle (Figure 8, right parcel). Preliminary clinical trials for the application of this technology have been performed and are under evaluation.
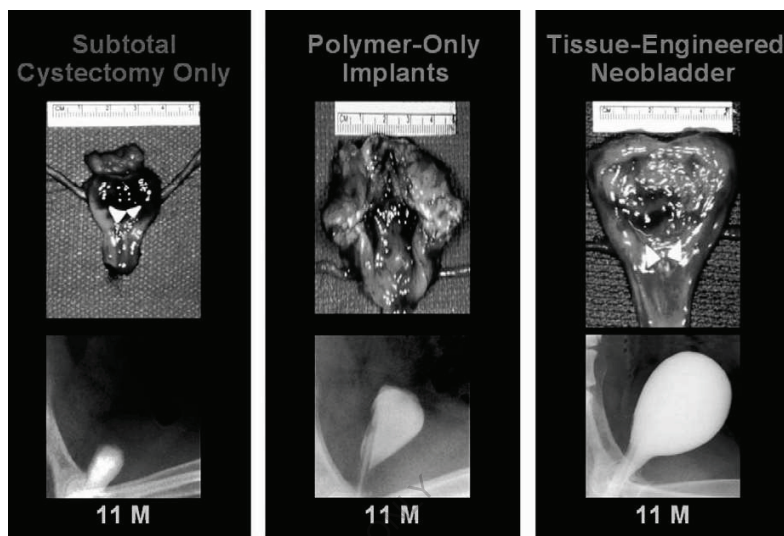
Figure 8. Comparison of tissue-engineered neobladders. Gross specimens and cystograms at 11 months of the cystectomy-only, nonseeded controls, and cell-seeded tissue-engineered bladder replacements. The cystectomy-only bladder had a capacity of 22% of the preoperative value and a decrease in bladder compliance to 10% of the preoperative value. The nonseeded controls showed significant scarring, with a capacity of 46% of the preoperative value and a decrease in bladder compliance to 42% of the preoperative value. An average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded tissue-engineered bladder replacements, and the compliance showed almost no difference from preoperative values, which were measured when the native sbladder was present (106%).

2.6.3 Genital Tissues

Tissue-engineering techniques have been used to reconstruct male and female genital tissues.

2.6.3.1 Corporal Smooth Muscle

Because one of the major components of the phallus is corporal smooth muscle, the creation of autologous functional and structural corporal tissue *de novo* would be beneficial. To examine functional parameters of engineered corpora, acellular corporal collagen matrices were obtained from donor rabbit penis, and autologous corpus cavernosal smooth muscle and endothelial cells were harvested, expanded, and seeded on the matrices. The entire rabbit corpora was removed and replaced with engineered scaffolds. The experimental corporal bodies demonstrated intact structural integrity by cavernosography and showed similar pressure by cavernosometry when compared with normal controls. The control rabbits (without cells) failed to show normal erectile function throughout the study. Mating activity in the animals with the engineered corpora appeared normal by one month after implantation.

The presence of sperm was confirmed during mating and was present in all the rabbits with the engineered corpora. The female rabbits mated with the animals implanted with engineered corpora and conceived and delivered healthy pups. Animals implanted with the matrix alone were unable to demonstrate normal mating activity and failed to ejaculate into the vagina.

2.6.3.2 Engineered Penile Prostheses

Although silicone is an accepted biomaterial for penile prostheses, biocompatibility remains a concern. The use of a natural prosthesis composed of autologous cells may be advantageous. A recent study using an autologous system investigated the feasibility of applying the engineered cartilage rods *in situ*. Autologous chondrocytes harvested from rabbit ear were grown and expanded in culture. The cells were seeded onto biodegradable poly-l-lactic acid–coated PGA polymer rods and implanted into the corporal spaces of rabbits. Examination at retrieval one month later showed the presence of well-formed, milky-white cartilage structures within the corpora. All polymers were fully degraded by two months. There was no evidence of erosion or infection in any of the implantation sites. Subsequent studies assessed the long-term functionality of the cartilage penile rods *in vivo*. To date, the animals have done well and can copulate and impregnate their female partners without problems.

2.6.3.3 Female Genital Tissues

Congenital malformations of the uterus may have profound implications clinically. Patients with cloacal exstrophy or intersex disorders may not have sufficient uterine tissue for future reproduction. We investigated the possibility of engineering functional uterine tissue using autologous cells. Autologous rabbit uterine smooth muscle and epithelial cells were harvested and then grown and expanded in culture. These cells were seeded onto preconfigured uterine-shaped biodegradable polymer scaffolds, which were then used for subtotal uterine tissue replacement in the corresponding autologous animals. Upon retrieval six months after implantation, histological, immunocytochemical, and Western blot analyses confi rmed the presence of normal uterine tissue components. Biomechanical analyses and organ bath studies showed that the functional characteristics of these tissues were similar to those of normal uterine tissue. Breeding studies using these engineered uteri are currently being performed.

Several pathologic conditions, including congenital malformations and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging due to the paucity of available native tissue. Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown, and expanded in culture. The cells were seeded onto biodegradable polymer scaffolds, which were then implanted into nude mice for up to six weeks. Immunocytochemical, histological, and Western blot analyses confi rmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, while the nonseeded structures were noted to be stenotic.

2.6.3.4 Ureter

Ureteral nonseeded matrices have been used as a scaffold for the ingrowth of ureteral tissue in rats. On implantation, the acellular matrices promoted the regeneration of the ureteral wall components. In a more recent study, nonseeded ureteral collagen acellular matrices were tabularized, but attempts to use them to replace 3-cm segments of canine ureters were unsuccessful. Cell-seeded biodegradable polymer scaffolds have been used with more success to reconstruct ureteral tissues. In one study, urothelial and smooth muscle cells isolated from bladders and expanded *in vitro* were seeded onto PGA scaffolds with tubular confi gurations and implanted subcutaneously into athymic mice. After implantation, the urothelial cells proliferated to form a multilayered luminal lining of tubular structures, while the smooth muscle cells organized into multilayered structures surrounding the urothelial cells. Abundant angiogenesis was evident. Polymer scaffold degradation resulted in the eventual formation of natural urothelial tissues. This approach has also been used to replace ureters in dogs.

2.6.4 Renal Structures

Due to its complex structure and function, the kidney is possibly the most challenging organ in the genitourinary system to reconstruct using tissue-engineering techniques. However, concepts for a bioartificial kidney are emerging. Some investigators are pursuing the replacement of isolated kidney function parameters with the use of extracorporeal units, while others are working toward the replacement of total renal function by tissue-engineered bioartificial structures.

2.6.4.1 *Ex Vivo* Renal Units

Although dialysis is currently the most prevalent form of renal replacement therapy, the relatively high rates of morbidity and mortality have spurred investigators to seek alternative solutions involving *ex vivo* systems. To assess the viability and physiologic functionality of a cell-seeded device to replace the filtration, transport, metabolic, and endocrinologic functions of the kidney in acutely uremic dogs, researchers introduced a synthetic hemofiltration device combined with a renal tubular cell therapy device (containing porcine renal tubules in an extracorporeal perfusion circuit). Levels of potassium and blood urea nitrogen (BUN) were controlled during treatment with the device. The fractional reabsorption of sodium and water was possible, and active transport of potassium, bicarbonate, and glucose and a gradual ability to excrete ammonia were observed. These results demonstrated the technologic feasibility of an extracorporeal assist device that is reinforced by the use of proximal tubular cells.

Using similar techniques, a tissue-engineered bioartificial kidney-consisting of a conventional hemofiltration cartridge in series with a renal tubule–assist device containing human renal proximal tubule cells- was used in patients with acute renal failure in the intensive care unit. Initial clinical experience with the bioartificial kidney and the renal tubule–assist device suggests that such therapy may provide a dynamic and individualized treatment program as assessed by acute physiologic and biochemical indices.

2.6.4.2 *In Vivo* Renal Structures

Another method of improving renal function involves augmenting renal tissue with kidney cell expansion *in vitro* and subsequent autologous transplantation. The feasibility of achieving renal cell growth, expansion, and *in vivo* reconstitution with tissue-engineering techniques has been explored. Most recently, an attempt was made to harness the reconstitution of renal epithelial cells to generate functional nephron units. Renal cells harvested and expanded in culture were seeded onto a tubular device constructed from a polycarbonate membrane connected at one end to a Silastic catheter terminating in a reservoir. The device was implanted into athymic mice. Histological examination of the implanted devices over time revealed extensive vascularization, with formation of glomeruli and highly organized tubulelike structures. Immunocytochemical staining confirmed the renal phenotype. Yellow fl uid consistent with the makeup of dilute urine in its creatinine and uric acid concentrations was retrieved from inside the implant (Yoo *et al*., 1996). Further studies using nuclear transfer techniques have been performed showing the formation of renal structures in cows (Figure 9). Challenges facing this technology include the expansion to larger, three dimensional structures.
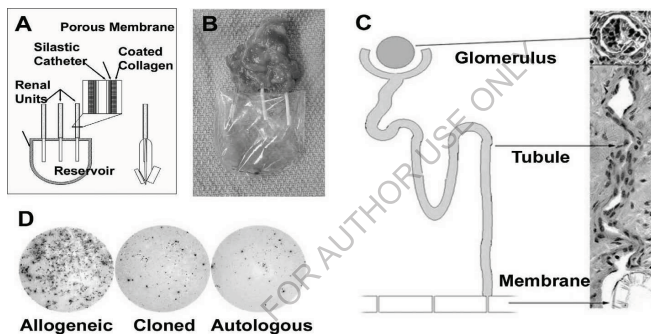
Figure 9. Creation of kidney tissue from therapeutic cloning and tissue-engineering strategies. (A) Illustration of the tissue-engineered renal unit. (B) Renal unit seeded with cloned cells, three months after implantation, showing the accumulation of urinelike fluid. (C) There was a clear unidirectional continuity between the mature glomeruli, their tubules, and the polycarbonate membrane. (D) Elispot analyses of the frequencies of T-cells that secrete IFN-gamma after primary and secondary stimulation with allogeneic renal cells, cloned renal cells, or nuclear donor fibroblasts.

3 Cell substrates and support materials

The value of a tissue engineering cell support depends on the information and suitability for the required adherent cell type or types. The information provided by the material is most commonly simple spatial cues, providing support surfaces with, for example, sufficient spacing for good cell growth. Biochemical information, such as surface reactive groups able to promote or reduce adherence or to activate cell membrane receptors (e.g., integrins) can be provided. More complex orientational and mechanical (tension, compression, or shear) information can be expressed through the supporting matrix of the construct.

The types of support materials available can be divided into discrete groups:
• Traditional: abiotic materials; metals, plastic, ceramics.
• Bioprostheses: natural materials modified to become biologically inert.
• Synthetic: resorbable polymers.
• Semi-natural: modified natural materials.
• Natural polymers: proteins, polysaccharides.

Composite devices can be constructed using more than one of these materials. For the purposes of tissue engineering constructs it is possible to largely omit the first group of traditional materials, because they are not designed to resorb or become biologically integrated within a reasonable period. Hence constructs based on these are more likely to be regarded as bioactive modifications of conventional prosthetic implants. In many cases, the same is also true of bioprosthetic materials in current use. These are materials formed by extensive chemical crosslinking of natural tissues, such as porcine heart valves and tendon. The native, collagen-based connective tissue is stabilized by glutaraldehyde treatment to produce non-immunogenic substrates which will survive largely unchanged at the implantation site for many years. Although cell layers and even new connective tissue can eventually grow over the surface of such bioprostheses *in vivo*, they have been designed and fabricated primarily to function as long as possible independently and without modification by surrounding host tissues. The resistance to cellular infiltration and remodelling of bioprostheses is counter to the basic aims of tissue engineering and largely discounts their use.

A variety of synthetic bioresorbable materials are degraded by hydrolysis and then phagocytosed. The advantage of such materials is that production is relatively cheap and easy, in a controllable and reproducible manner at large scale. Disadvantages lie in their cell compatibility, which is often not as good as for natural polymers, and their degradation products, which can have unwanted cellular effects.

Polymer composition is critical. Varieties include PGA, PLA, polycarbonate, poly e-caprolactone. The most widely applied polymers in tissue engineering are PGA and PLA and co-polymer PLGA. The composition, dimensions, and formation of these polymers can be adjusted to control their survival *in vivo* (stability), their gross mechanical properties (important in surgical handling and in replacing structural function), as well as their ability to support cell growth. In the case of PLGA this control is through adjustment of the ratios of PLA and PGA. PGA is crystalline, hydrophilic polyester, typically losing its mechanical strength through hydrolysis over two to four weeks. In contrast, PLA is more amorphous and hydrophobic, degrading to release lactic acid and losing mechanical strength after eight weeks, though with much slower total resorption *in vivo*. PGA, PLA, and PLGA have been used to support cells in a range of tissue engineering models including cartilage, urothelium, smooth muscle, and skin. The format of the support material is also important for different TE applications and relatively simple to modify using these polymers. The solid polymer screws and pins used in orthopaedics are less applicable to TE but early forms of polymer sutures were easily woven to give meshes and braids with porosities suitable for cell growth. Non-woven foams are made by incorporation of salt crystals into the polymer casting and subsequent dissolution by aqueous washing. Pore sizes are controlled by the size of the seeded crystals, typically between 150-300 μm. These materials can be moulded and extruded into a range of shapes including tubes for nerve guides. Semi-natural and natural substrates are derived from natural macromolecular polymers or whole tissues. The distinction between them lies in the extent to which materials are modified (to achieve

aggregation or stabilization) and how much this leads to frank denaturation. The most critical and pertinent test for TE applications is the extent to which a material participates in the natural remodelling process with local cells.

An example of a chemically crosslinked polysaccharide is mammalian hyaluronan, stabilized by benzyl esterification of increasing numbers of side chains. Hyaluronan is a charged polysaccharide (glycosaminoglycan) found naturally at cell surfaces and in the extracellular matrix. It is an important lubricant between gliding surfaces in soft tissues and has been implicated in a range of cellular functions, including angiogenesis and tissue repair. This aggregate, known as HYAFF (marketed as 'laser skin™'), is progressively more stable as crosslinking increases, but less of the material is biologically native and functional. Materials can be made with varying levels of substitution and these survive for progressively longer period's *in vivo*, eliciting only modest inflammation and local connective tissue response. HYAFF has been developed for use in skin grafting, particularly as a carrier for keratinocytes. Collagen sponges are prepared from various forms of insoluble or aggregated collagen, acid extracted, and crosslinked with agents such as carbodiimide, tannic acid or diphenylphosphorylazide. Some of these treatments produce substrates which can be utilized by cells *in vivo* and *in vitro*. A method for aggregating basement membrane into a cell substrate, termed TypeIV/TypeIVox, has been described using oxidatively crosslinked collagen purified from human placenta. It was designed principally to support growth of epithelial and endothelial cell layers and tested for the repair of skin and dura.

The most natural polymer materials are those in which protein stabilization is produced by drastic dehydration. This maximizes intermolecular interaction within polymers through complete removal of hydration shells, to promote intimate molecular packing. The most widely studied of these materials is the collagen-chondroitin sulfate aggregate material, which is available as 'Integra'. A range of forms have been tested with different added matrix components.

Aggregates of plasma fibronectin, produced as aligned fibrous materials, are derived from native protein solutions under directional shear and stabilized by dehydration. Their stability and attachment properties can be altered by treatment with trace levels of copper salts. For example, trace levels of copper differentially regulated growth and migration of Schwann cells and fibroblasts. This raises the possibility of producing modified substrates which have cell selectivity, potentially useful in segregating cell types between anatomical zones or layers.

The most natural forms of cell support are polymers whose aggregation can be achieved in culture as it occurs *in vivo*. Examples include Matrigel, fibrin glue, collagen gels, and some polysaccharides. Of the polysaccharides, chitosan and hyaluronan have been used in the form of a hydrated gel. Agarose gels are used to support a chondrogenic phenotype in chondrocytes. Matrigel is derived from tumour cells as a thick extracellular matrix, containing type IV collagen, laminin, proteoglycans, and growth factors. Despite being a natural substrate, its complex composition and tumour cell origin are limitations. There are commercially available forms of fibrin glue or fibrin sealant designed for surgical use. These are prepared from fibrinogen and thrombin from human plasma, stabilized by a bovine protease inhibitor, aprotinin. One composite form, Neuroplast, comprising elastin and fibrin, has been proposed as a substitute for dura mater and tympanic membranes. Fibrin materials have also been used as implantable growth factor depots and agents promoting angiogenesis,

forming a simple slow release depot for trapped agents. Growth factor depot formulation in fibrin stabilizes FGF activity.

Collagen gels or 'lattices' were developed to study contraction as the gel shrinks or tension is generated by fibroblasts. Untethered lattices shrink, producing smaller and denser matrices, sometimes regarded as tissuelike materials, whilst tethered lattices generate substantial endogenous forces. Collagen gels have been seeded with other cell types, such as keratinocytes and endothelial cells. Numerous model tissues have been attempted, based on contracted collagen gels as bioartificial grafts for skin replacement. The most advanced application is a bilayered tissue engineered allograft, 'Apligraf™' based on a fibroblast contracted lattice and keratinocyte layer. Orientated forms of collagen lattice have been developed to provide spatial information to cells either mechanically or more deliberately, using magnetic fields to provide contact guidance cues.

4 Cell sources

The first requirement for cell culture in any TE application is to generate sufficient cells to seed. The stage at which cells are to be introduced into the implant, the amount of remodelling of the implant substrate required, the degree of function, and the timing are critical.

One of the most important questions which must be addressed is whether the source of cells should be autologous, allogeneic, or xenogeneic. The use of the patient's cells (autologous transplant) is often the most straightforward option. A biopsy of the appropriate tissue is taken from the patient, enzymatically digested or explant cultured, and the cells grown to the required numbers. Whilst this method has the advantage of avoiding the immune response and the possible transfer of infection inherent in the use of allogeneic cells, it does have its drawbacks. Depending on the type of tissue required and the condition of the patient (limiting factors here include disease state and age of patient), it may not be possible to obtain adequate biopsy material. The cell type may pose problems, for example articular cartilage, which has a relatively low density of cells with a low mitotic capacity. Cosmetic issues influence the choice of skin for tissue engineering. Studies using keratinocytes derived from the sole of the foot indicate that the implanted cells resume their original phenotype and continue to produce a thickened epidermis. Genetically altering cells may alleviate this problem, but more needs to be known about the origins and maintenance of these regional cell differences within tissues.

Allogeneic sources of cells have advantages over autologous cells. It can be relatively easy to obtain healthy donor tissue; the cells may be cultured on a large scale at central 'factory' sites, with none of the time constraints of autologous cells. The product will be cheaper, of a consistent quality, and available as and when required by the patient. The major problem associated with allogeneic cells is graft rejection. The level of immune response generated by cells differs between cell types. For example, endothelial cells can induce a large reaction whilst fibroblasts, keratinocytes, and smooth muscle cells are less immunogenic. The reaction may also be dependent upon the donor age of the cells used, as fetal or neonatal cells can elicit little or no immune response. There have been many studies reporting methods for reducing the immunogenicity of transplanted cells, though with limited success. The current strategies involve segregation of the donor cells from the host using cell encapsulation techniques which allow only the passage in of oxygen and metabolites and the exit of cell-generated hormones. There must also be scrupulous staged analyses of cells for

possible infections, such as hepatitis, HIV, and CJD, as well as screening for potential hazards, such as abnormal karyotype, or tumorigenic capacity.

Gene therapy has a role to play in tissue engineering. Cell populations can be altered by adding genes either to increase their output of a certain protein or add to their repertoire of expressed genes. Examples include the alteration of keratinocytes to produce transglutaminase-1, which is deficient in patients with lamellar icthyosis, a disfiguring dermal disorder. The engineered cells were transplanted in athymic mice and produced a normal epidermis. The technique has also been used to induce fibroblasts and keratinocytes to release factors they would not normally produce. Fibroblasts have been made to produce proteins such as transferrin, factor IX, and factor VIII. In the vascular system modified endothelial cells have been used to produce antithrombogenic factors such as tissue plasminogen activator. Chondrocytes have been engineered to produce an antagonist for IL-1, a major cause of degradation of cartilage ECM.

The method of culture of cells for TE implants will depend on the function of the cells. For many cell types, the classical monolayer (or equivalent large scale culture in roller bottles or spinners) may allow proliferation of cells, but also increases the possibility of phenotypic changes. Many cells revert to a fibroblast-like morphology after long-term monolayer culture (e.g., chondrocytes) or if allowed to become too confluent may lose their proliferative capacity (e.g., endothelial cells). This change may result in a loss of function due to the inability to produce, for example, a certain hormone or the capacity to contract. It must be assessed whether this alteration is temporary, i.e., cells revert to original phenotype when replaced in a 3D format or under the influence of soluble factors *in vivo*, or if the changes are irreversible. In either case it may be preferable to culture cells in an environment similar to the *in vivo* situation.

Perfusion is a critical issue in 3D culture. A number of factors interact to determine when nutrient and gaseous perfusion of a cell mass become limiting, including:
- cell density
- metabolic rate
- mean effective diffusion distance (cell to mixed nutrient source)
- density and anisotropy of diffusion path material

Culture medium is exhausted more quickly by near-confluent cultures whilst some cell types, for example chondrocytes, survive at nutrient levels which would be critical to other cell types. In many experimental models, cells (e.g., fibroblasts or chondrocytes) grow in low density 3D aqueous gels. Collagen gels typically start at 95-98% water and these need to contract to less than 20% of their starting volume before they approach the cell and matrix densities of native tissues. Diffusion into such low density gels is rapid. In addition, 3D collagen lattices have a random, homogeneous fibril structure almost never encountered in native collagenous tissues. The native tissues have organized and aligned fibrous structures which will produce pronounced anisotropic diffusion properties. In particular, diffusion will be far more rapid parallel to rather than perpendicular to aligned collagen fibre bundles. As matrix density and alignment are characteristics of mature rather than repair tissues, perfusion will be a greater issue in TE of mature tissues. The principal means of addressing this problem to date has been to incorporate a form of external pumping to generate a fluid flow around or through the tissue construct. Throughout this section it is assumed that adequate perfusion of the construct will also optimize the rate of growth and the deposition of ECM. Control cues can also be optimized by adding growth factors and cytokines. Examples are the

use of mitogenic growth factors to promote fibroblast proliferation (PDGF and FGF) or VEGF or FGF to optimize endothelial cell proliferation. Similarly, rates of matrix production may be regulated by 'control cues' such as the TGFp and IGF families of growth factor. However, the incorporation of specific growth factors is extremely cell system- and stage-specific and this is made more complex by the need for multi-component cocktails.

5 Orientation

Cells require specific shape, directional, and spatial cues from their environment including:
- contact or substrate guidance
- chemical gradients
- mechanical cues

In its simplest form, contact guidance uses the topographical features of the substrate. Topographical features are most frequently in the form of aligned fibres or ridges of appropriate dimensions (normally < 100 μm for fibres). Cell types which are aligned in this way include fibroblasts, tenocytes, neurites, macrophages, and Schwann cells. Bioresorbable guidance templates suitable for tissue engineering are based on synthetic polymer substrates, collagen fibrils and fibronectin. Alignment of collagen fibrils within native collagen gels has utilized mechanical or magnetic forces, whilst tethering points, holes or defects in the gel and the local effects of contractile cells can produce local orientation. Orientation of fibronectin fibres involves application of directional shear as part of the fibre aggregation process. Figure 10 shows a form of orientated fibronectin cable with a surface layer of aligned dermal fibroblasts, which migrate rapidly along the structure. It is possible to use differential attachment to substrates as a means of producing alignment in cells.

The speed of cell locomotion is cell type- and substrate-dependent, as demonstrated by the ability to alter cell speed on more or less adhesive substrates. Fibroblast speeds of 20-40 μm/h are common. It is possible to optimize cell speeds by suitable design of the substrate composition but a far greater impact is made on recruitment by optimizing cell velocity. Random direction of movement will have a minimal velocity until some guiding cue is provided. In the case of cell guidance substrates, such as fibronectin fibre substrates or patterned resortablc polymers, cell speed and persistence of motion can be linked with directional cues to optimize the cellular velocity.

Figure 10. Scanning electron micrograph showing human dermal fibroblasts aligned on a fibronectin cable (alignment top to bottom).

Delivery of spatial or directional cues to cells in the form of chemical gradients through 3D constructs requires that the chemical agent not only stimulates the cell function, but also that it is presented to cells as a directional gradient. A number of growth factors are chemotactic and certain extracellular macromolecules, such as fibronectin and collagen, produce chemotactic fragments on breakdown. Such diffusible agents can be important in tissue organization. Angiogenesis, for example, is based on the concept that new vessels move along gradients of diffusible factors. These can be towards sites of low oxygen tension or high lactate concentration, or towards local sources of growth factor production. For example, vascular endothelial cell growth factor (VEGF) or FGF or matrix components such as oligosaccharide fragments of hyaluronan influence angiogenesis. Whilst such gradients may be useful in special circumstances, their use in control of architecture is likely to be limited in *vitro*. The most plausible circumstances would be for:

1. Control over short ranges (e.g., between distinct cell layers in a tissue construct).
2. During early stages of matrix development and maturation.

Use of hydrated 3D matrices for support of cultured cells, based on collagen, fibrin or fibronectin is helpful in minimizing convection mixing of gradients. However, as discussed earlier there are likely to be major practical difficulties in producing and maintaining effective gradients in a controlled manner as the matrix becomes dynamic, dense and anisotropic.

5.1 Mechanical cues

A wide range of cell types are sensitive to mechanical loading. The mechanisms by which cells respond to mechanical signals are complex and include modulation of cytosolic-free calcium, stretch-sensitive ion channels and deformation of cytoskeletal or integrin components. Responses can include changes in cell alignment, synthesis of active regulatory molecules such as growth factors or hormones, altered matrix synthesis, and enzyme release. There are at least three different forms of mechanical cue: tension, compression, and shear. Fibroblasts are normally considered in terms of tensional forces, vascular endothelial cells predominantly under fluid shear and chondrocytes are adapted to compression forces. It is possible to further differentiate each of these forms of mechanical cue, for example into

cyclical and static force or on the basis of loading velocity. However, our understanding of the effects of these forms of loading or their combinations is limited.
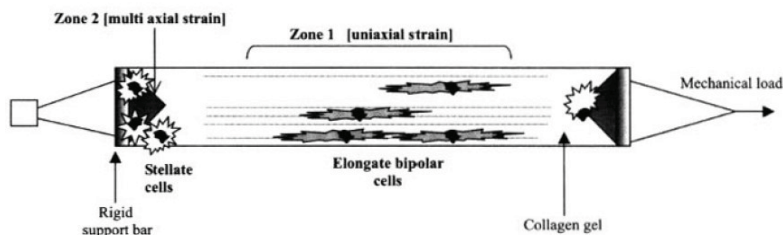
Figure 11. Schematic illustration of fibroblast alignment produced in collagen gels by uniaxial loading, Strain was aligned parallel with the long axis of the gel (and applied load in zone 1) and cells here took on an elongate, bipolar morphology, parallel to the strain. Strain in zone 2 was poorly aligned (represented by the dark triangle) due to the shielding effect of the rigid support bars and cells here were stellate in shape showing no alignment.

A further, and potentially critical complexity, is that mechanical stimuli used in *in vitro* experimental models frequently have little or no directional component. Systems which give either non-orientated, or highly complex, multiple direction cues can give little information on the control of architecture and tissue polarity. They are valuable primarily for investigation of the role of mechanical forces on tissue production, turnover, and composition. A number of shear force models are being developed for tissue engineering of tube tissues such as blood vessels. Fluid shear forces have been found to operate through cytoskeletal changes, cell-cell adhesion sites and protein kinase and G protein signalling in vascular endothelial cells. These pathways are reported to affect heat shock protein phosphorylation, Cu/Zn superoxide dismutase activity and apoptosis. Alteration of extracellular matrix production and cell shape and alignment are more likely to be important for microarchitecture control cues. Current examples of tissue engineered tube structures for use in fluid drainage are bioartificial blood vessels and bladder.

The ability of fibroblasts to become orientated along a uniaxial mechanical load affects their pattern of matrix remodelling. Similar orientations have been reported in loaded skeletal muscle cells. Multi-axial complex loading patterns, produced by the flexcell analytical model, produces complex cell alignments, which are difficult to interpret, though collagen production is quantitatively increased by loading. In uniaxial, cyclically loaded collagen lattices, fibroblasts take on an alignment parallel with the maximum strain induced in the material at any given location. This has been used in one experimental model to identify two distinct zones of mechanical loading in the same gel (Figure 11).

Most studies of compressional loading have used chondrocytes in a 3D agarose gel matrix or whole cartilage. The agarose matrix provides little or no attachment until chondrocytes have elaborated their own pericellular matrix. In addition, agarose is essentially homogeneous in structure, providing uniform mechanical support in all dimensions. Consequently, newly synthesized matrix is easily detected and applied loads act uniformly and predictably on most cells. In contrast, cartilage in organ culture contains cells in a non-homogeneous matrix with mechanically distinct zones.

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Chapter 9
Cytotoxicity and viability assays

1 Introduction

Drug development programmes involve pre-clinical screening of vast numbers of chemicals for specific and non-specific cytotoxitity against many types of cells. Both are important for indicating the potential therapeutic target and safety evaluation. Animal models have always played an important role in both contexts, and although cell culture systems have figured largely in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing is now widely accepted, there is increasing pressure for a more comprehensive adoption of *in vitro* testing in safety evaluation. The impetus for change originates partly from financial considerations, because *in vitro* testing has considerable economic advantages over *in vivo* testing. There is also an increasing realization of the limitations of animal models in relation to human metabolism, as more and more metabolic differences between species come to be identified. Finally and as importantly, there is the moral pressure to reduce animal experimentation.

The safety evaluation of chemicals involves a range of studies on mutagenicity, carcinogenicity, and chronic toxicity. Whilst the major application of *in vitro* cultures is the analysis of acute toxicity, the existence of adequate culture systems would also improve the prospects of chronic toxicity testing. Toxicology and cancer chemotherapy therefore share the aim of determining the acute toxicity of a range of chemicals against a variety of cell types. In both areas there may be several ultimate goals:

• identification of potentially active compounds
• identification of the mechanism by which the compound exerts its toxic effect
• prediction of anticancer activity
• identification of a potential target cell population
• identification of the toxic concentration range
• relationship of concentration to exposure time (C x T)

The fundamental requirements for both toxicology and screening for anticancer activity are similar. They are first that the assay system should give a reproducible dose-response curve with low inherent variability over a concentration range which includes the exposure dose *in vivo.* Secondly, the selected response criterion should show a linear relationship with cell number, and thirdly, the information obtained from the dose-response curve should relate predictively to the *in vivo* effect of the same drug.

2 Background

Use of in *vitro* assay systems for the screening of potential anticancer agents has been common practice almost since the beginnings of cancer chemotherapy in 1946, following the discovery of the antineoplastic activity of nitrogen mustard.

Accumulated experience, both clinical and experimental, demonstrated the heterogeneity of chemosensitivity between tumours, even those of identical histology. The successful development of the *in vitro* agar plate assay for antibiotic screening precipitated interest in the development of an analogous technique for 'tailoring' chemotherapy to suit the

individual rumour and patient, thus removing the undesirable combination of ineffective chemotherapy in the presence of non-specific toxicity.

The correlation between *in vitro* drug sensitivity exhibited by primary cultures of human tumours and their *in vivo* counterparts argued for their use in drug evaluation programmes, because they provided a closer approximation to the human clinical situation than did the limited number of cell lines which were used.

The National Cancer Institute now routinely measures the growth inhibitory properties of every compound under test against a panel of 60 human tumour cell lines which are representative of major human tumour types. For each compound tested, the GI50 (concentration of drug needed to inhibit cell growth by 50%) is generated from the dose-response curves for each cell line. The pattern of all the cell lines as a group is then used to rank compounds according to whether a new compound is likely to share a common mechanism of action with compounds already present in the database (COMPARE). In recent years, the COMPARE computer algorithm has been applied to correlate patterns of growth inhibition with particular molecular properties of the cell lines. The developments in the field of safety evaluation of drugs have been reviewed by several authors. There are a number of advantages *of in vitro* testing for safety evaluation which include analysis of species specificity, feasibility of using only small amounts of test substances, and the facility to do mechanistic studies.

3 Specific techniques

The choice of assay depends on the context in which the assay is to be used, the origin of the target cells, and the nature of the test compounds. Parameters which vary between assays include:
• culture method
• duration of drug exposure and drug concentration
• duration of recovery period after drug exposure
• end-point used to quantitate drug effect

3.1 Culture methods

The choice of culture method depends on the origin of the target cells and the duration of the assay and, to some extent, dictates the end-point.

3.1.1 Organ culture

The advantages of organ culture relate to the maintenance of tissue integrity and cell-cell relationships *in vitro,* giving a model that is more representative of the *in vivo* situation than the majority of culture methods available. However, reliable quantitation of drug effect is impaired by variation in size and cellular heterogeneity between replicates. Although the method has been used extensively to study the hormone sensitivity of potentially responsive target tissues, the number of studies relating to drug sensitivity is limited.

3.1.2 Spheroids

Spheroids result from the spontaneous aggregation of cells into small spherical masses which grow by proliferation of the component cells. Their structure is analogous to that of a

small tumour nodule or micrometastasis, and the use of spheroids for drug sensitivity testing therefore permits an *in vitro* analysis of the effects of three-dimensional relationships on drug sensitivity, without the disadvantages previously mentioned for organ culture. Specific parameters which can be studied are:
• drug penetration barriers in avascular areas
• the effects of metabolic gradients (e.g., nutrients, metabolites, pO_2, pCO_2)
• the effects of proliferation gradients

The majority of studies have been carried out using spheroids derived from cell lines, but primary human tumours also have the capacity to form spheroids in approximately 50% of cases. The spheroid-forming capacity of normal cells is limited in comparison with tumour cells, so stromal elements may be excluded during reaggregation of human tumour biopsy material. Culture times in excess of two weeks are usually necessary for drug sensitivity testing, and the method is therefore not suitable in a situation where results are required quickly.

3.1.3 Suspension cultures
i. *Short-term cultures (4-24 hours)*

The short-term maintenance of cells in suspension for assay of drug sensitivity is applicable to all cell sources. When the cells are derived from human tumour biopsy material, the assay system has several theoretical advantages in that stromal cell overgrowth and clonal selection are minimized, and results can be obtained rapidly. The method has been used extensively in Germany for chemosensitivity studies on a variety of tumour types. A modified method using either tissue fragments or cells has been described, again with a variety of tumour types using the incorporation of tritiated nucleotides into DNA or RNA as an end-point. Limitations of the method relate mainly to the short time period of the assay, which precludes long drug exposures over one or more cell cycles and takes no account of either the reversibility of the drug's effect or of delayed cytotoxicity.

ii. *Intermediate duration cultures (4-7 days)*

Suspension cultures of intermediate duration are particularly suited to chemosensitivity studies on haematological malignancies, and have been described in several reports.

3.1.4 Monolayer culture

The technique of growing cells as a monolayer has been most frequently applied to the cytotoxicity testing of cancer cell lines, but the method has also been used with some success for studies on the chemosensitivities of biopsies from a variety of different tumour types. In the case of human biopsy material the greatest problems associated with the method are, first, that the success rate is limited because adherence and proliferation of tumour cells is not always obtained and, secondly, that contamination of tumour cell cultures by stromal cells may occur. Some method of cell identification is therefore an essential part of such assays. The technique has also found wide acceptance with toxicologists because appropriate cell lines may be available for the development of models for specific organ systems.

Monolayers offer great flexibility in terms of drug exposure and recovery conditions, and in methods of quantitation of drug effect. The range of microtitration plates now available together with developments in automation has in turn led to microscale

methodologies which are economical in terms of cell numbers required and reagents. There have been major developments in this area in recent years facilitating high throughput drug screening using cell lines. When cell numbers from biopsies are limited it may be feasible to culture the cells until sufficient numbers are available for assay, although reports on changes in chemosensitivity after subculture are conflicting. Two to three subcultures are probably acceptable and, indeed, subculture has been recommended because variability between replicates is reduced. When stromal cell contamination is unacceptably high, subculturing also offers the possibility of 'purifying' tumour cell cultures by differential enzyme treatment or physical cell separation.

3.1.5 Clonogenic growth in soft agar

Although monolayer cloning can be applied to cells cultured directly from the tumour, the majority of reports in recent years have used suspension cloning to minimize growth of anchorage-dependent stromal cells. Clonogenic assays have the theoretical advantage that the response is measured selectively in cells with a high capacity for self-renewal (stem cells). However, this is only true if colonies are clones (i.e., were initiated from one cell and not from a clump) and are scored after many cell generations in clonal growth. Cloning efficiencies of 0.01-0.1% may be obtained, but it is difficult to exclude the possibility of clumps; whilst ten generations (about 1000 cells) can be readily obtained in monolayer cloning, suspension colonies are often scored after four to six cell generations (16-64 cells). Given that some of these colonies started out as clumps of three or four cells or larger, the generation number could be as low as two and their capacity for self-renewal in doubt.

Technical problems have been encountered using solid tumours and effusions from patients, which unfortunately influence interpretation of results. These include difficulties in obtaining a pure single cell suspension from epithelial tumours, very low plating efficiencies (<< 1%), the formation of colonies from anchorage-dependent cells under certain growth conditions, requirements for large cell numbers, and finally the somewhat subjective nature of colony quantitation. Critical assessment of the technical difficulties which have been encountered with the 'human tumour stem cell assay' can be found in several reports. An alternative methodology, developed by Courtenay and others gives higher plating efficiencies, and in a comparison with the 'Hamburger-Salmon' system it was apparent that the methodology used influenced the chemosensitivity profile obtained (Figure 1).

In recent years comparatively few publications have dealt with the applied use of the assay, but there have been several reports describing methods of improving plating efficiencies in soft agar.
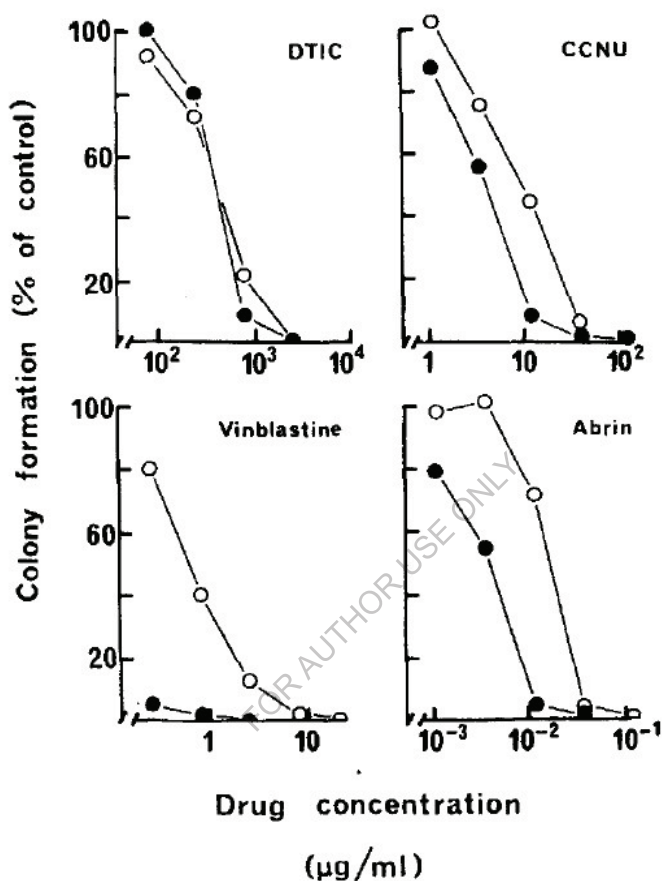
Figure 1. Dose-response curves of a melanoma xenograft cultured in soft agar using the 'Courtenay' method (•) and the 'Hamburger-Salmon' method (O). Cells were exposed to the drugs for 1 h, plated at 3×10^4 cells per tube or dish, and scored for colonies after 14 days incubation. Control cultures produced about 400 colonies using both methods.

3.2 Duration of drug exposure and drug concentrations

The choice of drug concentrations should be dictated by consideration of the therapeutic levels which can be achieved with clinically used drug dosages. When the compound is undergoing pre-clinical screening for potential antitumour activity this is not possible. Accumulated evidence on effective *in vitro* levels of compounds with known *in vivo* activity suggests an upper limit of 100 μg/ml.

Pharmacokinetic data are available for many of the clinically used anticancer drugs, and parameters which are relevant to *in vitro* assays include the peak plasma concentration and the plasma clearance curves (Figure 2). A detailed description of pharmacological considerations may be found elsewhere. Pharmacokinetic data on most cancer chemotherapeutic agents, which includes peak plasma concentration, the C X T parameter (where C is concentration and T is time in hours), and the terminal half-life ($t_{1/2}$) of the drug in plasma, have been summarized. When no pharmacokinetic data are available, an approximation of the plasma levels can be obtained by calculation of the theoretical concentration obtained when the administered dose is evenly distributed throughout the total body fluid compartment. It is axiomatic that the concentration range adopted should give a dose-response curve. The same provisos apply when deciding on appropriate drug concentrations for the assessment of acute toxicity, though upper concentration levels may be in excess of 100 µg/ml.

Pharmacokinetic data show that maximum exposure to drug occurs in the first hour after intravenous injection and, for this reason, an exposure period of 1 h has been chosen by many investigators. Whilst this may be adequate for cycle-specific drugs, such as the alkylating agents, longer exposure times over several cell cycles may be necessary for phase-specific drugs. Prolonged drug exposure using a variety of cancer chemotherapeutic agents has been shown to result in gradually decreasing ID_{50} values, as exposure times increase (figure 3). In some cases these reach a stable minimum plateau value. Rate of penetration of the drug may also be a limiting factor when short exposure times are used. Ultimately the question of duration of drug exposure becomes one of practicality. If a significant effect is achievable in 1 h, then this should be used. Many drugs may bind irreversibly to intracellular constituents and the actual exposure may therefore be in excess of 1 h due to drug retention. Others, principally the antimetabolites and antitubulins, are more likely to be reversible if not present at the sensitive phase of the cell cycle, and prolonged exposure spanning one or more cell cycles may be required. Resistance of the surviving fraction when short exposures are used may be due to the phase of the cell cycle during drug exposure, but genetically resistant cells (i.e., with resistance even at the appropriate phase of the cell cycle) can only be demonstrated unequivocally after a prolonged exposure. When prolonged drug exposure times are used, it should be remembered that the theoretical C X T value is only equal to the actual C X T value when the drug retains full activity at 37 °C over the entire exposure period and the response is linear with time. Data on the stability of some anticancer drug solutions at 37 °C have been reported. The effective concentration of drug may also be reduced by binding of drug to the surface of the incubation vessel and medium components. Considerations such as those described above are also relevant for assessment of the acute toxicity of a compound.
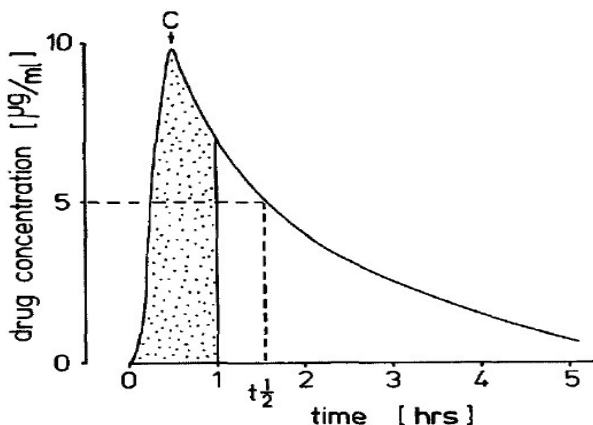
Figure 2. Typical plasma clearance curve for intravenously administered drug. C, peak plasma concentration; $t_{1/2}$, terminal half-life of drug in plasma. The hatched area is the area under the curve for T = 1 h.
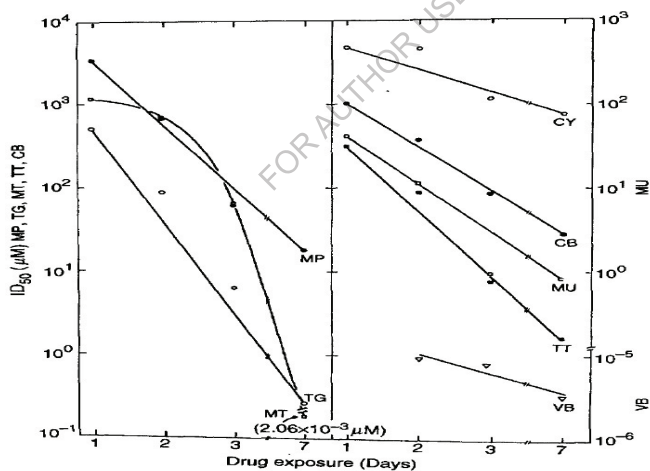
Figure 3. Effect of prolonged drug exposure on ID_{50} values for 6-mercaptopurine (MP), thioguanine (TG), methotrexate (MT), cyclophosphamide (CY), chlorambucil (CB), mustine (MU), thiotepa (TT), and vinblastine (VB), tested against HeLa cells using a microtitration plate assay. Drugs were replaced at 24 h intervals for the 48 h and 72 h exposures, and at 24, 48, and 72 h for the 7 day exposure. A break in the time axis is therefore shown between 3 and 7 days.

3.3 Recovery period

The inclusion of a recovery period following drug exposure may be important for three reasons:

    a.   When metabolic inhibition is used as the end-point, it allows the cells to recover from metabolic perturbations which are unrelated to cell death.

    b.   Sublethal damage can be repaired and therefore not interfere with the assay result.

    c.   Delayed cytotoxicity, perhaps not expressed until one to two cell cycles after drug treatment, can be measured.

    d.

Depending on the drug and the end-point, absence of a recovery period can either underestimate or overestimate the level of cell kill achieved. However, it is equally important that the recovery period is not too long, because cell kill can then be masked by overgrowth of a resistant population. In monolayer assays which monitor cell counts or precursor incorporation, the cells must remain in the log phase of growth throughout the exposure and recovery period.

4 End-points
4.1 Cytotoxicity, viability, and survival

Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death, whereas survival assays measure the end-result of such metabolic perturbations, which may be either cell recovery or cell death. Theoretically, the only reliable index of survival in proliferating cells is the demonstration of reproductive integrity, as evidenced by clonogenicity. Metabolic parameters may also be used as a measure of survival when the cell population has been allowed time for metabolic recovery following drug exposure.

4.2 Cytotoxicity and viability

Some cytotoxicity assays offer instantaneous interpretation, such as the uptake of a dye by dead cells, or release of [51]Chromium or fluorescein from pre-labelled cells. These have been termed tests of viability, and are intended to predict survival rather than measure it directly. On the whole these tests are good at identifying dead cells but may overestimate long-term survival. Most imply a breakdown in membrane integrity and irreversible cell death. Other aspects of cytotoxicity, measuring metabolic events, may be more accurately quantified and are very sensitive, but prediction of survival is less certain as many forms of metabolic inhibition may be reversible. In these cases impairment of survival can only be inferred if depressed rates of precursor incorporation into DNA, RNA, or protein are maintained after the equivalent of several cell population doubling times has elapsed.

4.2.1 Membrane integrity

This is the commonest measurement of cell viability at the time of assay. It will give an estimate of instantaneous damage (e.g., by cell freezing and thawing), or progressive damage over a few hours. Beyond this, quantitation may be difficult due to loss of dead cells by detachment and autolysis. These assays are of particular importance for toxic agents which exert their primary effect on membrane integrity.

i. [51]Chromium release

Labelling cells with ^{51}Cr results in covalent binding of chromate to basic amino acids of intracellular proteins. These labelled proteins leak out of the cell when the membrane is damaged, at a rate which is proportional to the amount of damage. The method is used in immunological studies for determining cytotoxic T cell activity against target cells. Natural leakage of ^{51}Cr from undamaged cells may be high, and therefore the time period over which the assay can be used is restricted to approximately 4 h. Additionally, the target cells must be prelabelled before incubation with drug or effector cells, and the final preparation for counting as well as counting itself is time-consuming. In one comparative study which evaluated ^{51}Cr release as an end-point for anticancer drug cytotoxicity testing the method was found to be of no value.

ii. *Enzyme release assays*

Enzyme release assays for measuring membrane integrity have been developed which overcome some of the problems of the ^{51}Cr release assay. Different enzymes have been used, though LDH has been found to be generally useful, because it is released by a range of cell types. This paper describes the assay for determining cytotoxicity of antibody-dependent and T cell-mediated reactions. The assay also has application in the wider context of toxicity testing, and has been used to investigate hepatotoxicity.
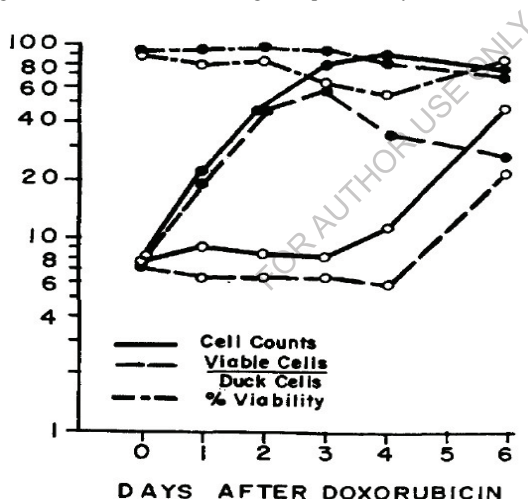
Figure 4. Effect of doxorubicin (0.12 µg ml-1.h) on MDAY-D2 cells as assessed by three different techniques: Coulter counter particle counts (-) *(y* axis units are cells/ml x 10^-4); ratio of living tumour cells to duck red blood cells, normalized to the same scale as the Coulter counts (- -), and percentage viability (---) y axis units are percentage viability (living cells/living and dead cells x 100). •, control cultures; O, doxorubicin-treated cultures.

iii. *Dye exclusion*

Viability dyes used to determine membrane integrity include trypan blue, eosin Y, naphthalene black, nigrosin (green), erythrosin B, and fast green. Staining for viability assessment is more suited to suspension cultures than to monolayers, because dead cells

detach from the monolayer and are therefore lost from the assay. A major disadvantage may be the failure of reproductively dead cells to take up dye, as demonstrated when cells with impaired clonogenicity showed 100% viability according to dye exclusion. The method has been renovated, however, and technical innovations introduced which attempt to circumvent some of the problems commonly associated with such assays.

Comparison of cell counts versus percentage viability versus viable cell/duck cell ratio demonstrated the increased sensitivity of the latter method (Figure 4). The method has been applied with equal success to solid tumours, effusions, and haematological malignancies.

iv. Fluorescent dyes

There are a number of fluorescent probes now available for measuring membrane integrity. These are shown in Table 1.

Table 1. Commercially available kits for oytotoxicity assays

Company	Kit	Method
Lumitech	Vialight	Bioluminescent detection of ATP.
Lumitech	Apoglow	Measures changes in ADP/ATP ratio.
Stratagene	Quantos Cell proliferation assay kit	Measures fluorescence of a dye when bound to DNA.
Sigma	MIT-based assay kit	Metabolism of tetrazolium salts to insoluble formazan products by mitochondrial enzymes.
Sigma	XTT-based assay kit	Metabolism of tetrazolium salts to soluble (XTT) formazan products by mitochondrial enzymes.
Sigma	Acid phosphatase-based assay kit	Uses p-nitrophenol phosphate as substrate.
Sigma	Neutral red-based assay kit	Neutral red taken up by lysosomes and Golgi bodies.
Sigma	Kenacid blue-based assay kit	Binds to protein.
Sigma	Sulforhodamine B-based assay kit	Binds to protein.
Sigma	Lactate dehydrogenase-based assay kit	Measures membrane integrity.
Sigma	Cell Census Plus™ system for cell proliferation analysis using flow cytometry	Cells pre-labelled with fluorescent dye. After drug exposure immunostaining can be used to detect drug effects on specific subsets of cells.

4.2.2 Respiration and glycolysis

Drug-induced changes in respiration (oxygen utilization) and glycolysis (carbon dioxide production) have been measured using Warburg manometry. Other authors have determined dehydrogenase activity by incorporating methylene blue into agar containing drug-treated cells, cell death being indicated by non-reduction of the dye. The latter method has the disadvantage of being non-quantitative, whilst the former, although quantitative, has not been widely adopted because the technical manipulations involved are extensive and unsuited to multiple screening. The more direct approach of monitoring pH changes in cultures containing an appropriate pH indicator has also been described.

4.2.3 Radioisotope incorporation

Measurement of the incorporation of radiolabelled metabolites is a frequently used end-point for cytotoxicity assays of intermediate and short-term duration.

i.   *Nucleotides*

Measurement of [³H]thymidine incorporation into DNA and [³H]uridine incorporation into RNA are commonly used methods of quantitation of drug cytotoxicity. In short-term assays, which do not include a recovery period, there are a number of disadvantages, all of which relate to a failure of [³H]thymidine incorporation to reflect the true DNA synthetic capacity of the cell. These are:

    a. Changes may relate to changes in size of the intracellular nucleotide pools rather than changes in DNA synthesis.
    b. Some drugs such as 5-fluorouracil and methotrexate which inhibit pyrimidine biosynthesis *(de novo* pathway) cause increased uptake of exogenous [³H]thymidine due to a transfer to the 'salvage' pathway, which utilizes preformed pyrimidines.
    c. Continuation of DNA synthesis in the absence of [3H]thymidine incorporation can occur.
    d.

The low labelling index of human tumours with resultant low levels of nucleotide incorporation in short-term assays necessitates the use of high cell densities, which can restrict the number of drugs and range of concentrations tested when cell numbers are limited. Two 'hybrid' techniques have been reported, which combine the advantages of the soft agar culture system with the facilitated quantitation offered by the use of radioisotopes. Both assays are of intermediate duration (about four days) and use [³H]thymidine incorporation into DNA as an end-point. In one method the cells are grown in liquid suspension over soft agar, whilst in the other the cells are incorporated in the soft agar. Given that a homogeneous cell population is available, [³H] nucleotide incorporation can be used after an appropriate recovery period to measure survival or, in the presence of drug, to measure an antimetabolic effect, but with the reservations expressed above.

ii. *[¹²⁵I]Iododeoxyuridine ([¹²⁵I]Udr)*

[¹²⁵I]Udr is a specific, stable label for newly synthesized DNA which is minimally reutilized and can therefore be used over a 24 h period to measure the rate of DNA synthesis; quantitation is facilitated because the isotope is a gamma emitter. Disadvantages include its variable toxicity to different cell populations, which therefore means that more cells are required because [¹²⁵I]Udr must be used at low concentrations.

ii.   *[³²P]Phosphate (³²P)*
iii.

The rate of release of ³²P into the medium from pre-labelled cells is a function of the cell type and is increased in damaged cells. This has been used as a measure of drug efficacy. The incorporation of ³²P into nucleotides has also been used as an index of drug cytotoxicity. Neither method has been routinely adopted.

iv. *[¹⁴C] Glucose*

Glucose incorporation is used as a cytotoxicity end-point because it is a precursor which is common to a number of biochemical pathways. The method has not been widely used.

v. [^{3}H] Amino acids

Protein synthesis is an essential metabolic process without which the cell will not survive, and incorporation of amino acids into proteins has been used as an index of cytotoxicity. The most extensive studies have utilized monolayers of cells growing in microtitration plates, using either incorporation of [^{3}H] leucine measured by liquid scintillation counting, or [^{35}S]methionine incorporation, measured using autofluorography.

vi. ^{45}Calcium (^{45}Ca)

Unrelated compounds may produce alterations in the permeability of cell membranes to calcium, such that increased calcium uptake results. Measurements of ^{45}Ca uptake can therefore be used.

4.2.4 Total protein content

Protein content determination is a relatively simple method for estimating cell number. It is particularly suited to monolayer cultures, and has the advantage that washed; fixed samples can be stored refrigerated for some time before analysis without impairment of results, facilitating large scale screening. Over estimation of cell number may arise with drugs which inhibit replication without inhibiting protein synthesis (e.g., BrdU, methotrexate). Assessment of cytotoxicity requires the demonstration of an alteration in the accumulation of protein per culture over time, preferably at several points, or at one point after prolonged drug exposure and recovery, as described above.

4.2.5 Colorimetric assays

The advent of sophisticated microplate readers which allow rapid quantitation of colorimetric assays has paralleled the development of a variety of assays which use some form of colour development as an end-point for quantitating cell number. These include methods which reflect:
- protein content (methylene blue, Coomassie blue, Kenacid blue, sulforhodamine B, Bichinoninic acid)
- DNA content (Hoechst 33342) or DNA synthesis (BrdU uptake)
- lysosome and Golgi body activity (neutral red)
- enzyme activity (hexosaminidase, mitochondrial succinate dehydrogenase)

Linear relationships between end-point and cell number have been demonstrated for all these methods. Discrimination between live and dead cells in monolayer assays is not relevant, because dead cells will usually detach, given sufficient time. In suspension cultures this aspect is relevant, however, and needs to be considered when choosing an appropriate assay. Methods which make use of fluorescent dyes have increased in the past five years and bioluminescent assays have also been developed for measuring ATP levels.

i. Protein content

Several methods are available for measuring the protein content of cell monolayers. These include the use of the Folin-Ciocalteau reagent according to the method of Lowry and amido black. Several new techniques for colorimetric determination of protein content are also available. These include methylene blue, sulforhodamine B, Kenacid blue, Coomassie blue G-250 and Bichinoninic acid (BCA).

ii. *DNA content*

DNA content may be measured in microtitration plates by staining with dyes whose fluorescence is enhanced by intercalation at AT-specific sites on the chromatin, such as Hoechst 33342 and 2-diamidinophenylindole (DAPI). DNA synthesis may also be determined using bromodeoxyuridine (BrdU). The amount of BrdU incorporated is detected immunohistochemically using a monoclonal antibody to BrdU, and the binding may be quantitated using appropriate conjugates and chromogenic substrates.

iii. *Lysosomal and Golgi body activity*

The uptake of neutral red by lysosomes and Golgi bodies has been used to quantitate cell number. The stain appears to be specific for viable cells, but the main limitation of the method is the difference in uptake between cell types. Thus, some cell types, such as activated macrophages and fibroblasts, take up large amounts very rapidly whereas others, such as lymphocytes, show negligible staining.

iv.   *Tetrazolium dye reduction*

The most widely used technique involves the use of a tetrazolium salt (e.g., MTT, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which is metabolized to an insoluble coloured formazan salt by mitochondrial enzyme activity in living cells. The method was first described in 1983 as a rapid colorimetric method for immunological studies, and modifications for this application have been described. The technique is particularly useful for assaying cell suspensions because of its specificity for living cells. One disadvantage is the need to use unfixed cells, which may impose time restraints. The potential of the technique for drug sensitivity testing of human tumours was recognized, and early reports of its use are promising. These include the possibility of increased mitochondrial enzyme activity in drug-treated cells and the effect of medium conditioning by cells on formazan production. The absorption spectrum of MTT-formazan has also been shown to be dependent upon pH and cell density and a method has been described which overcomes these problems and increases linearity between MTT-formazan and cell density especially at high cell numbers. Another derivative, XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) produces a soluble formazan end-product and is therefore easier to use than MTT because there is no need to carry out the solubilization procedure. The XTT derivative has been used successfully for quantitating colony formation in soft agar.

v. *Luminescence-based cell viability testing*

Levels of ATP in a cell population provide a sensitive indication of cell viability. The luminescence reaction used to determine ATP levels is based on the following reaction.

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$$\text{ATP} + \text{D-luciferin} + O_2 \xrightarrow[\text{Mg}^{2+}]{\text{Luciferase}} \text{AMP} + 2P + CO_2 + \text{light}$$
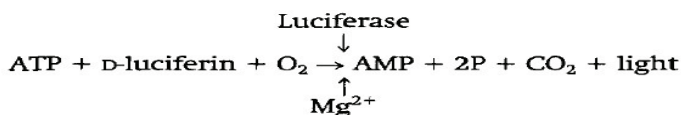
The sensitivity range has been reported as 20 cells/ml to 2×10^7 cells/ml. A number of commercial kits are available for measuring ATP by luminescence (Table 2).

v. *Apoptosis*

Many anticancer drugs kill cells by apoptosis and measurement of apoptosis is therefore important in the evaluation of cytotoxicity. Apoptosis can be determined in a number of ways including:

- morphological criteria
- DNA laddering
- detection of phosphatidyl serine in the outer plasma membrane using annexin V conjugated to FITC or biotin (Tables 1 and 2)

Table 2. Fluorescent probes used to investigate membrane integrity

Dye	Characteristics
Calcein-AM	Membrane-permeant esterase substrate; cleaved by esterase in living cells to yield calcein, which fluoresces green in the cytoplasm.
Ethidium-homodimer	High affinity red fluorescent nucleic acid stain which penetrates membrane of dead but not live cells.
Propidium iodide	Red fluorescent nucleic acid stain which penetrates membrane of dead but not live cells.
DiOC18	Green fluorescent membrane stain which can be used in combination with propidium iodide for cell-mediated cytotoxicity assays studying membrane integrity.
Chromatide™ BODIPYF®L-14-dUTP (Molecular Probes)	Fluorescent labelled nucleotide used in TUNEL assay.
Annexin V-FITC	Annexin V binds to phosphatidyl serine and fluorescent label allows demonstration of transfer of phosphatidyl serine from inner to outer plasma membrane which is a feature of apoptosis.
CyQUANT™ GR (Molecular Probes)	Proprietary green fluorescent dye which shows enhanced fluorescence when bound to cellular nucleic acids

4.3 Survival (reproductive integrity)

Survival assays give a direct measure of reproductive cell death by measuring plating efficiency either in monolayer or in soft agar. The end-points described in the previous section can also be used as an index of reproductive integrity providing the design of the assay incorporates a recovery period.

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4.3.1 Cloning in monolayer

Cells generally have a higher cloning efficiency in monolayer than in soft agar. Normal cells and tumour cells will form colonies in monolayer, and the method is not therefore applicable to tumour biopsy material, unless criteria are available to discriminate between tumour and non-tumour cells. Feeder layers of irradiated or mitomycin C-treated cells can be used to increase plating efficiencies, and indeed small drug-resistant fractions are more likely to be detected in the improved culture conditions existing when feeder cells are used.

4.3.2 Spheroids

Various methods can be used to quantitate the effect of drugs on spheroid growth. These are:

- relative changes in volume of treated and untreated spheroids
- cloning efficiency in soft agar of disaggregated spheroids
- cell proliferation from spheroids adherent to culture surfaces

The first method is rather insensitive because spheroid growth tends to plateau, and the second may be affected by difficulties with disaggregation to a single cell suspension and low plating efficiencies.

4.3.3 Cell proliferation

Growth curves may be determined and the doubling time during exponential growth derived. An increase in doubling time is taken as an indication of cytotoxicity, but it must be stressed that this is a kinetic measurement averaged over the whole population and cannot distinguish between a reduced growth rate of all cells and an increase in cell loss at each cell generation. Estimates of cytotoxicity based on cell growth in mass culture must utilize the whole growth curve, or they may be open to misinterpretation. If 50% of cells die at the start of the experiment, the growth rate of the residue, determined in log phase, may be the same, but will show a delay. In practice, it is difficult to distinguish between early cell loss and a prolonged lag period. Cell growth rates must be taken as only a rough guide to cytotoxicity, and accurate measurements of cell survival and cell proliferation should be made by colony-forming efficiency (survival) and colony size (proliferation).

5 Assay comparisons

In spite of the diversity of methodologies used for cytotoxicity and viability testing, approximately the same levels of correlation between *in vitro* sensitivity and *in vivo* response have been reported when the methods have been applied to human tumour biopsy material. A number of comparative studies have been undertaken, which generally indicate that appropriately designed cytotoxicity assays give results which are comparable to clonogenic assays. The relative merits of clonogenic and non-clonogenic assays have been discussed and reviewed.

6 Interpretation of results
6.1 Relationship between cell number and cytotoxicity index

The validity of a cytotoxicity index is dependent upon the degree of linearity between cell number and the chosen end-point, and this should be confirmed for any cytotoxicity

assay. In clonogenic assays a linear relationship may not occur at low cell numbers due to the dependence of clonogenic growth on conditioning factors, whilst at high cell densities linearity is lost due to nutritional deficiencies. In cytotoxicity assays linearity may be lost at the upper end due to density-dependent inhibition of the relevant metabolic pathway, whilst the sensitivity limit of the assay may affect linearity at the lower end. This would cause apparent stimulation at low drug levels and an overestimation of cell kill at higher concentrations. Control cell numbers at the end of the assay must therefore fall on the linear portion of the curve. The accuracy at higher levels of cell kill is dependent on the range over which linearity extends, and influences the number of decades of cell kill which can be measured. If results are plotted on a log scale this may imply that the assay is accurate down to three or four decades of cell kill, which should be confirmed before expressing results in this way. This is particularly important in *in vitro* drug combination studies when synergism or additivity is often observed beyond the second decade of cell kill.

7.2 Dose-response curves

Results are commonly plotted as dose-response curves using a linear scale for percentage inhibition (of isotope incorporation, for example) and a log scale for surviving fraction on clonogenic tests. Assay variation for replicate points is routinely depicted as mean ± standard deviation; a minimum of three replicates is therefore required for each test point. Some method is required for defining the sensitivity of a cell population in relation to other cell populations, or different test conditions; several parameters are available and are shown in Figure 6.

7.2.1 Area under curve method

The use of this method acknowledges the probability that the shape of the dose-response curve may be instrumental in influencing the outcome of drug exposure, rather than cell kill at any one concentration. It is calculated using the trapezoidal method which adds the area of rectangles and triangles under the survival curve. The method has been applied most extensively in the 'human tumour stem cell assay'. Some drugs do not produce curves with an easily defined IC_{50} value and AUC may provide a more easily quantifiable result.

7.2.2 Cut-off points for definition of sensitivity and resistance

If plots of dose-response curves from comparisons of cell lines show that they maintain their relative sensitivity rankings at different concentrations (i.e., crossing-over of dose-response curves is minimal), then information on the relative sensitivities of different cell lines can be obtained by defining sensitivity at one concentration, and this is the most commonly used method for *in vitro* predictive testing. When retrospective correlations between *in vitro* data are made for defining these cut-off points, an intermediate zone is found where tumours cannot be defined as sensitive or resistant, and there is no clear-cut correlation between *in vitro* results and clinical response. The size of the intermediate zone will be at least partly related to the inherent variability of the assay, larger zones being associated with higher standard deviations. Although sensitivity may be defined at one concentration it is recommended that more than one concentration is tested, particularly in the developmental stage of the assay.

7.2.3 ID_{50} and ID_{90} values

Cell sensitivity may also be defined by the ID_{50} and ID_{90} values (i.e., drug concentration required to inhibit viability by 50% or 90%). These values may also be termed IC (inhibitory concentration), LC (lethal concentration), or GI (growth inhibition), but they are all determined in the same way.

7.2.4 Correlation between *in vitro* and *in vivo* results

Criteria for defining tumours as sensitive or resistant are based on retrospective correlations between *in vitro* results and clinical responses, using a training set of data. Even when a laboratory is using an established method for tumour sensitivity testing, 'own laboratory' sets of training data should be obtained to allow for inter-laboratory variation. The response of patients with tumours of intermediate sensitivity may be influenced by prognostic factors other than tumour sensitivity (e.g., tumour burden at onset of chemotherapy, stage of disease, histology, tumour cell doubling time, previous chemotherapy, and performance status). When analysing results for correlations, some attempt to stratify patients according to these parameters may assist in providing more meaningful data. Quantitative assessment of tumour response is also of paramount importance. It is pointed out that *in* vitro chemosensitivity can be expected only to indicate that some degree of cell kill will be achieved *in vivo*, not that the patient will achieve a complete response to treatment. The true positive correlation rate of an assay is defined as:

$$\frac{S^t/S^p + S^t/R^p}{S^t/S^p}$$

where the numerator of each fraction is the *in vitro* test response (t) and the denominator is the *in vivo* patient response (p). S^t and R^t are respectively the number of tumours showing in vitro sensitivity or resistance according to the selected cut-off points. S^p and R^p are respectively the number of patients achieving a clinical response or no clinical response to the drug tested *in vitro*. The true negative rate is defined as:

$$\frac{R^t/R^p + R^t/S^p}{R^t/R^p}$$

In assessing the significance of the correlation rates obtained, these should be compared with the correlation rates which would be obtained were the *in vitro* results randomly distributed. For example, a drug gives a 50% response rate *in vivo,* and 50% of tumours show *in vitro* sensitivity to this drug. If the *in vitro* results are randomly distributed between sensitivity and resistance, then the chances of obtaining a positive correlation between *in vitro* sensitivity and *in vivo* response are 50% of 50% (i.e., 25%), and of obtaining a positive correlation between *in vitro* resistance and *in vivo* resistance. The overall apparent positive correlation rate is therefore 50%. *In vitro* versus *in vivo* correlations are also complicated by the use of combination regimes to treat patients. Strictly speaking, correlations should be made only when *in vitro* data is available for all drugs used. Whether or not they are tested in combination depends on the treatment protocol, because some drugs are administered sequentially. Also, if the assay can only measure two decades of cell kill it may be too insensitive to detect additive or synergistic effects.

7.2.5 Combinations of drugs

A 96-well plate clonogenic assay based on limiting dilutions has been used to look at additive and supra-additive (synergistic) effects of drug combinations. The data is analysed

using the formula plating efficiency (PE) = -In (number of negative wells)/(total number of wellsj/number of cells plated per well. Fractional survival data are then fitted to the linear quadratic model,

$$F = \exp[-(O_tD + (\beta D2)]$$ and AUC analysed by numerical integration using appropriate software.

8 Pitfalls and troubleshooting

Problems which may be encountered with these assays include:
- large standard deviations
- variability between assays done on the same cell population
- stimulation to above control levels

8.1 Large standard deviations
Possible reasons for large standard deviations include:
- Faults in aliquoting cell suspension, which are most likely to be made due to inadequate mixing of cell suspension during dispensing leading to uneven distribution of cells between replicates.
- The presence of large cell aggregates in the original cell suspension, leading to uneven distribution of cells between replicates.
- Non-specificity of cytotoxicity end-point (e.g., due to measurement of nonspecific binding of radioactivity.

8.2 Variation between assays

Replicate assays on different days cannot be performed on human tumour biopsy material to check day-to-day reproducibility, but this can be evaluated using cell lines. It is a recognized problem that cell lines which show consistent sensitivity profiles may show 'deviant' results occasionally, for reasons which cannot be identified. Specific reasons for failure to obtain reproducible results may include:

- Failure to harvest the cell population at an identical time point (e.g., exponential growth versus early confluence versus late confluence).
- Deterioration of stock drug solutions.
- When drug solutions have a short half-life they must be used immediately after diluting to ensure consistency in the drug levels available to cells in each assay.
- Failure to standardize incubation conditions.

The assay system must be checked for reproducibility before applying it to human biopsy material.

8.3 Stimulation to above control levels

Stimulation can be a true measure of cellular events but may be due to technical artefacts. These include:

(a) Non-specific binding of radioactivity.
(b) Density-dependent inhibition of metabolic pathways in controls which is not evident in test situations where some cell kill has been achieved.

(c) Stimulation of uptake of metabolic precursors by antimetabolites (e.g., thymidine by 5-fluorouracil and methotrexate).
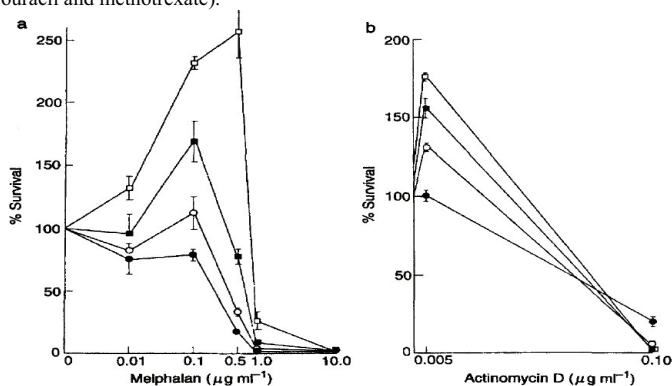
Figure 5. The effect of growth unit size on the survival curves of a murine melanoma cell line (CCL) to melphalan (a) and of a human melanoma biopsy to actinomycin D (b). Growth unit size and frequency was measured using FAS II automated image analysis system. •, - 60 um; O, - 104 u*m;* •, a 124 urn; D, a 140 (um. Mean ± s.e. shown.

Plated cell density influences the distribution in size of growth units in clonogenic assays, with large units decreasing as plated cell numbers increase. The effect of this on drug sensitivity profiles was examined and, as expected, the dose-response curve was strongly influenced by the size criterion used for colony scoring, with stimulation to above control levels occurring when large colonies were scored (Figure 5).

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Chapter 10

Genetic Engineering

1 Introduction

To explore the role of a gene, we need to analyse its regulation and expression during and after development, the function of its product, and interactions with other proteins. In most cases, it is necessary to be able to transfer the gene, or its manipulated form, into cells. However, as mammalian cells do not take up foreign DNA efficiently, the availability of effective methods for introducing genes into the cells is essential. Many gene transfer methods have been developed that are routinely used by scientists studying mammalian cells. Moreover, with the advancement of recombinant DNA technology, gene transfer techniques have become powerful tools in gene cloning and mapping, construction of transgenic animals, and gene therapy.

In this chapter some of the most commonly used methods for introducing genes into mammalian cells, including transfection by calcium phosphate precipitation and cationic lipids, electroporation, and the techniques of microcellmediated chromosome transfer and irradiation fusion gene transfer, will be discussed. Other techniques of gene delivery, which are not discussed here, include DEAE dextran neutralization, microinjection of DNA directly into the cells and the use of viral vectors.

Due to their versatility and efficiency, viral vectors have become attractive tools for the introduction of genes into human cells in gene therapy studies. Their drawbacks are the biological hazards associated with viruses and in some cases the immunogeniciry of viral vectors. Alternative therapeutic gene delivery systems use cationic lipids as carriers of the exogenous DNA.

2 Transfection

Gene transfer methods by transfection can be classified into two major categories: biochemical and physical. The biochemical methods include calcium phosphate precipitation and lipid-mediated transfections, and physical transfection is achieved by electroporation. The choice of method depends on the cell type. For example, haematopoietic cell lines are more efficiently transfected by electroporation than the biochemical based methods, whereas some cell types take up DNA efficiently when it is associated with cationic lipids rather than calcium phosphate precipitates. Conversely, cationic lipids can be highly toxic to some other cell types.

Following transfection, the exogenous DNA remains in the nucleus for a limited time unless it is integrated into the host genome. However, even in the non-integrated form, the transfected DNA is subject to the regulatory mechanisms that control gene expression and therefore its genes can be expressed transiently. This transient gene expression has been exploited in studies such as the analysis of regulatory sequences and optimization of transfection conditions. Integration of the transfected gene into the host genome, Conversely, gives rise to stable retention of the exogenous gene in the cells, providing constitutive expression.

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In most transfection experiments the cDNA of the gene of interest is inserted into a mammalian expression vector that carries an expression cassette consisting of a strong promoter, such as the LTR promoter of Rous sarcoma virus (for expression of the gene in mammalian cells), a multiple cloning site, and a polyadenylation sequence. The vector also contains a bacterial origin of replication and a bacterial selectable marker (for propagation and selection of the vector in bacterial cells). To isolate the few transfected mammalian cells from the majority of non-transfected ones, a dominant mammalian selectable marker is also utilized. This marker may be on a different vector that is cotransfected into the cells along with the vector or DNA sequence that carries the target gene. In this case, although the co-transfected DNAs are not physically linked, a considerable number of them integrate into the genome in large concatemeric structures that can be up to 2000 kb. Therefore it is believed that at some stage after entering the cell co-transfected DNA sequences are ligated to each other before integrating into the host genome. Transfection is a powerful tool for cloning genes that give rise to phenotypic effects in the transfected cells. This gene cloning strategy, however, requires the rescue of the exogenous DNA from the genomes of the cells that exhibit the phenotypic effects. Several procedures have been described to achieve this, but they are often cumbersome and involve second rounds of transfections followed by construction of genomic libraries from the secondary transfectants and screening of this library for the cloned gene. Hence, other types of vectors have been generated that utilize the Epstein-Barr virus (EBV) origin of replication. The EBV origin of replication allows these vectors to replicate in human cells during each cell division while maintained in an episomal state (i.e., non-integrated). Therefore these vectors have two advantages. First, they give rise to greater transfection efficiencies, because they do not need to integrate to be maintained, and secondly, following transfection, they can be shuttled back into an *E. coli* host, thus making their subsequent isolation easier. The EBV-based shuttle vectors were successfully used in cloning the human cDNAs for Fanconi's anaemia complementation group C (FACC) and xeroderma pigmentosum group C (XPCC) genes.

2.1 Calcium phosphate-DNA co-precipitation

The method of calcium phosphate-DNA co-precipitation is the most widely used technique for the transfection of mammalian cells. This technique was first introduced by Graham and Van der Eb in 1973. Since then there have been many modifications and optimization protocols for obtaining higher transfection frequencies for both stable and transient expressions of exogenous genes. The principle of this technique is the formation of insoluble calcium phosphate-DNA complexes in a supersaturated solution. This is achieved by adding a solution containing DNA and $CaCl_2$ to a buffered saline solution containing phosphate and incubating the mixture for a period of time, to allow the formation of calcium phosphate-DNA co-precipitates. The mixture is added to culture cells in medium followed by incubation at 37°C for a period, usually between 6-24 hours, depending on the cell type. DNA molecules enter the cells by endocytosis of the calcium phosphate-DNA co-precipitates. Several of the factors that influence the efficacy of this method are described below.

2.1.1 DNA

The concentration, topology, and quality of DNA affect the transfection efficiency. It has been shown that suboptimal concentrations of DNA drastically reduce the efficiency of calcium phosphate transfection. In agreement with this, studies on the kinetics of the formation of calcium phosphate-DNA complex indicate that high DNA concentrations inhibit

this process and lead to reduction of DNA associated with the insoluble complex. Moreover, Chen and Okayama have reported that supercoiled plasmid DNA gives rise to several-fold higher numbers of stable transformants than linearized DNA.

2.1.2 pH

Optimum pH range for the formation of calcium phosphate precipitates for most cell lines is 6.95 to 7.05. It has been suggested that the CO_2 level in the incubator during transfection also influences the transfection frequency.

2.1.3 Standing and incubation times

Standing time greatly affects the quality of precipitates for transfections. The longer the calcium phosphate-DNA mixture is incubated before addition to the cells the coarser the precipitates will become, and as there is an optimal size of the precipitates to be taken up by cells, fine-tuning is needed to obtain the best standing time. It appears that the solubility of calcium phosphate is reduced by increasing the temperature; however, most protocols suggest room temperature and varying standing times of 1-20 minutes. Incubation time for transfection varies between a few hours up to 16 hours, depending on the cell type.

2.2 Gene transfer
2.2.1 Lipid-mediated gene transfer (lipofection)

The use of cationic lipids for DNA transfection into mammalian cells has become widespread because several features of these reagents make them attractive vehicles for gene delivery, particularly in gene therapy. For instance, they are safer than viral vectors, can be produced in large quantities, and can deliver large DNA fragments of up to several megabase pairs long into cells.

There are many formulations of lipid reagents for transfection, but they normally contain a positively charged moiety attached to a neutral lipid component. The first generation of lipid reagents for transfection contained neutral lipids that relied on methods for capturing DNA molecules within the liposomes, but today's cationic lipids form lipid-DNA complexes that make the transfection efficiency independent of encapsulation of DNA. On mixing of these reagents with DNA, the charged head groups are drawn towards the phosphate backbone of DNA and form lipid-DNA complexes. When the suspension of these complexes is added to the cells, the positively charged head groups of the lipid are attracted to the negatively charged cell membrane. The end result is that the lipid-DNA complex is either fused to the cell membrane or enters the cell by endocytosis, transferring its DNA load into the cell. It is due to these properties of the cationic liposomes that efficient DNA delivery has been achieved in many cell lines.

Various liposome formulations are commercially available but it is important to bear in mind that the transfection efficiency depends very much on the cell type and the chemical and physical structure of the cationic liposomes, particularly with respect to the liposome size and cationic head group. Thus preparations must be compared to identify the formulation that gives the best transfection efficiency for the cell line of interest. Other significant factors in using these reagents for DNA transfection are lipid:DNA ratio, their concentrations, DNA quality, cell culture density, and the duration of exposure of cells to the lipid.

2.2.2 Viral mediated gene transfer

a. Retroviral Vectors

Basics of the retrovirus virion and infection. Retrovirus virions contain a protein capsid that is lipid encapsulated. Virions range in diameter from 80 to 130 nm. The viral genome is encased within the capsid along with the proteins integrase and reverse transcriptase. The genome consists of two identical positive (sense) single-stranded RNA molecules ranging in size from 3.5 to 10 kilobases. Following cellular entry, the reverse transcriptase synthesizes viral DNA using the viral RNA as its template. The cellular machinery then synthesizes the complementary DNA which is then circularized and inserted into the host genome. Following insertion, the viral genome is transcribed and viral replication is completed. The majority of retroviruses are oncogenic although the degree to which they cause tumors varies from class to class.

Retroviral receptors. Retroviruses of cats and mice are typically classified by host range. This has led to the use of the following termonology. Ecotropic viruses are viruses which use receptors unique to mice and are only able to replicate within the murine species. Xenotropic viruses uses receptors found on all cells in most species except those of mice. Polytropic and amphotropic viruses use different receptors found in both murine and nonmurine species.

The retroviral genome. The retroviral genome consists of little more than the genes essential for viral replication. The prototype and simplest genome to describe is that of the Moloney murine leukemia virus (MMLV) in contrast to the highly complex genomes of the HTLV and HIV retroviruses. The genome can be divided into three transcriptional units: gag, pol and env. The gag region encodes genes which comprise the capsid proteins; the pol region encodes the reverse transcriptase and integrase proteins; and the env region encodes the proteins needed for receptor recognition and envelope anchoring. An important feature of the retroviral genome is the long terminal repeat (LTR) regions found at each of the gene. The LTR plays an important role in initiating viral DNA synthesis and its integration as well as regulating transcription of the viral genes.

MMLV genome:

Retroviral Vectors for Gene Transfer : MMLV

MMLV vectors. To date, these vectors have been used more than any other gene transfer vehicle. They are produced simply by replacing the viral genes required for replication with the desired genes to be transferred. Thus, the genome in retroviral vectors will contain an LTR at each end with the desired gene or genes in between. The most commonly used system for generating retroviral vectors consists of two parts, the retroviral DNA vector and the packaging cell line.

Retroviral DNA vectors are plasmid DNAs which contain two retroviral LTRs in the region internal to these LTRs for insertion of the desired gene. A portion of a retroviral plasmid DNA vector, LNSX, is shown below.

The gene of interest is cloned into the multicloning site following the simian virus SV40 promoter (SV). As one can imagine, these plasmid DNAs can be manipulated to meet a variety of needs allowing for multiple applications and the design of very elegant vectors.

Packaging cell lines provide all the viral proteins required for capsid production and the virion maturation of the vector. These packaging cell lines have been made so that they contain the gag, pol and env genes. Early packaging cell lines contained replication competent retroviral genomes and a single recombination event between this genome and the retroviral DNA vector could result in the production of a wild type virus. This led to the term "helper virus contamination" and to this date, with all viral vectors systems, it is important to insure that the vector be free of helper virus. Current packaging systems require that three homologous recombination events occur for any wild type virus production. As this is an extremely rare event, current cell lines are considered to be helper free, although it is still wise to test for contamination of any wild type virus. There have been a wide variety of packaging cell lines produced as well as retroviral DNA vectors, and most are commercially available. For the MMLV vectors it is the packaging cell line which determines if the vector is ecotropic, xenotropic or amphotropic. Dependent upon the target cells it is important to ensure that the correct packaging cell line be chosen.

Vector preparartion. Following insertion of the desired gene into in the retroviral DNA vector, and maintainance of the proper packaging cell line, it is now a simple matter to prepare retroviral vectors. The retroviral DNA vector is transferred into the packaging cell line using calcium phosphate mediated transfection, a procedure which we will describe in later lectures. After approximately two days for virion production, the virus is harvested, and this virus is then used to infect a second packaging cell line. Doing this will allow you to produce a virus with a variety of host ranges. The flow chart below is a quick schematic of vector production.

224
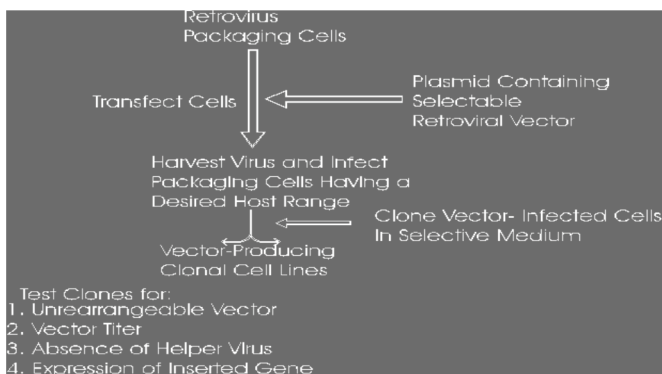
Human immunodeficiency viruses (HIV) are the most recently discovered members of the retrovirus family and have led to the new classification of lentivirus. Like other members of the retroviral family, the HIV genome contains the gag, pol and env genes. In addition, several other nonstructural proteins which serve regulatory functions are contained within its genome. Below is the diagram of the HIV genome considered to be one of the most complex among the retroviruses. Notice that the basic genome units (gag, pol and env) and LTRs predominate the genome.
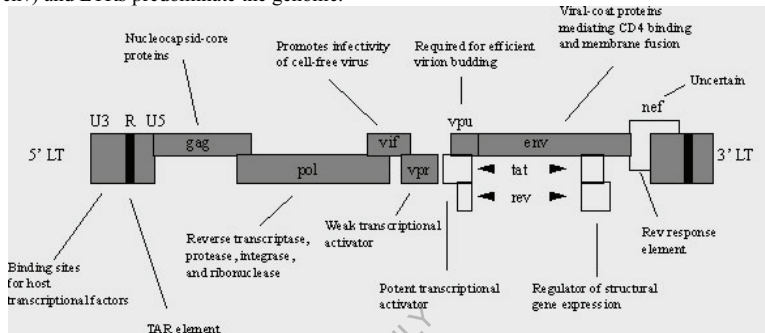
Potential applications. Rather than an in depth focus on HIV biology, we will discuss the current applications of the HIV virus in gene therapy. HIV-based vectors are recent developments in the field of gene therapy and focus on treatment of AIDS. Vector production is similar to that of MMLV vectors, and HIV vectors possess all the same advantages and disadvantages as their MMLV counterparts. An exciting application of HIV vectors is to use HIV vectors to target genes selectively into HIV-containing cells. The goal is that genes delivered by the HIV vector would allow selective killing of any cell previously or subsequently infected with HIV. An example of how this might be accomplished focuses on the **tat** and **rev** genes found in the HIV genome. These genes are also found only in cells infected with HIV. By using a tat and rev induced promoter to drive the expression of a toxin gene, any cell infected with HIV should be selectively killed upon expression of the toxin gene. Of course this system does require that a cell become infected with HIV but once infected the cell would terminate as would the HIV virus. A major concern in using HIV vectors is the fact that there is a strong possibility for genetic recombination between infectious HIV and the HIV vector itself. Such an event would result in the HIV vector acting as an infectious HIV particle. One possible method to overcome this risk would be the inclusion of a suicide gene into the HIV vector genome. The suicide gene would make cells infected with the HIV vector genome sensitive to a drug that would poison only those cells containing the HIV vector genome. An example of a suicide gene is the Herpes simplex virus thymidine kinase gene, and ganciclovir is the drug which would yield selective toxicity.

Table 1 . Advantages and disadvantages of retroviral vectors

Advantages	Disadvantages
High Transduction Effciency	Requires dividing cells for infectivity
Insert Size up to 8kB	Low Titers (10^6- 10^7)
Integrates into host genome resulting in sustained expression of vector	Integration is random
Extremely well studied system	*In vivo* delivery remains poor. Effective only when infecting helper cell lines
Vector protiens not expressed in host	

b. Adenoviral Vectors

Adenoviruses were discovered in 1953 as investigators hurriedly attempted to identify the causative agents of the common cold. There are currently 47 distinct serotypes and as many as 93 different particular varieties of adenovirus, all of which generally infect the ocular, respiratory or GI epithelium. In 1977, Frank Graham developed a cell line which enabled the first production of recombinant adenoviruses in a helper free environment. Since this time, adenoviral vectors have recevied much attention as gene transfer agents and currently offer a wide variety of gene therapy applications.

Basic structure of the adenovirus virion. Adenoviral virions are icosahedral in shape, 70 to 90 nm in diameter, and are not enveloped. The viral capsid contains 252 protein components the majority of which are three proteins: **fiber, penton based** and **hexon**. Fiber and penton base proteins are important in receptor binding and cell internalization, whereas hexon comprises the majority of the viral capsid. The viral genome is large, consisting of a single double-stranded DNA molecule 36 to 38 kilobases in size. Viral DNA replication and transcription are complex, and viral replication and assembly occur only in the nucleus of infected cells. Mature virions are released by cellular disintegration.

Adenoviral infection is a highly complex process. It is initiated by the virus binding to the cellular receptor. Internalization occurs via receptor-mediated endocytosis followed by release from the endosome. After endosomal release, the viral capsid undergoes disassembly as it journeys to the nuclear pore. Nuclear entry of the viral DNA is completed upon capsid dissociation, and the viral DNA does not integrate into the host genome but remains in an episomal state.

Replication deficient adenoviruses. As adenoviral replication depends on the E1A region of the viral genome, all recombinant adenoviral vectors have this region of its genome deleted, and are referred to as "replication-deficient". Such vectors are capable of infecting a cell only once, no viral propagation occurs, and the infected cell does not die as a result of vector infection.

Adenoviral vector production. Recombinant adenoviral vectors are prepared from two components: viral DNA vector and a packaging cell line. The adenoviral DNA vector is a plasmid DNA that contains a portion of the viral genome. It has had the E1A region deleted, and desired genes are cloned into a multicloning site that has been inserted in place of the E1A region of the genome. Unfortunately the large size of the adenoviral genome pervents the use of a single plasmid-based system in vector production. Instead, the adenoviral vector is produced using either *in vitro* ligation or homologus recombination. Using *in vitro* ligation,

wild-type adenoviral DNA is isolated and cleaved with the restriction endonuclease ClaI. The digested viral DNA is then ligated onto the adenoviral DNA vector containing the gene of interest which has previously been digested with ClaI. The ligated DNA species is then transfected into the packaging cell line. The homologous recombination system can use either ClaI digested viral DNA or a plasmid containing all the adenoviral sequence downstream from the ClaI site. Both the adenoviral DNA vector and the viral DNA component are co-transfected into the packaging cell line. These DNA species are then left to undergo homologous recombination within the cells resulting in vector production. The diagram below illustrates both the *in vitro* ligation and homologus recombination protocols.
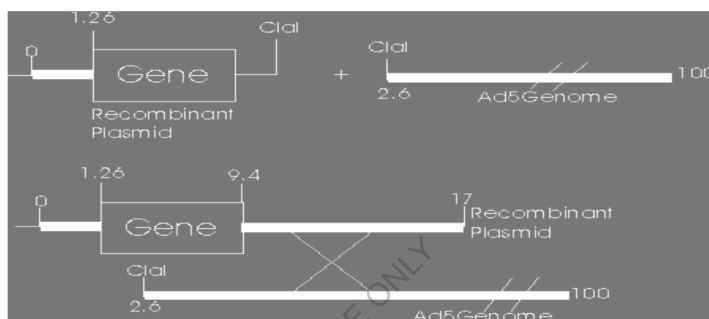
Vector production. The 293 cell line is utilized for viral production. This is a human kidney cell line which has been stably transfected with the E1A region of the adenoviral genome. This allows the vector to be made and matured within the 293 cell, yet vectors prepared from this cell line will lack the E1A region and remain replication-deficient. As with any viral vector system, one must ensure that the vector produced is free of wild-type contamination. In the adenoviral system, at least two homologous recombination events must occur to obtain wild type virus.

Advantages and disadvantages. Adenoviral vectors have a high transduction efficiency, are capable of containing DNA inserts up to 8 kilobases, have extremely high viral titers (on the order of 1010-1013), and infect both replicating and differentiated cells. Also, as they lack integration, they can not bring about mutagenic effects caused by random integration into the host genome. Disadvantages of adenoviral vectors include the following: (1) expression is transient because the viral DNA does not integrate into the host, (2) viral proteins are expressed in the adenoviral vector following administration into the host, and (3) adenoviral vectors are an extremely common human pathogen and *in vivo* delivery may be hampered by prior host immune response to one type virus.

Table 2. Advantages and disadvantages of adenoviral vectors

Advantages	Disadvantages
High transduction efficiency	Expression is transient (viral DNA does not integrate)
Insert size up to 8kb	Viral protiens can be expressed in host following vector administration
High viral titer (10^{10}-10^{13})	*In vivo* delivery hampered by host immune response

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Infects both replicating and differentiated cells	

c. Lentiviruses

Lentiviruses are a subclass of Retroviruses. They have recently been adapted as gene delivery vehicles (vectors) thanks to their ability to integrate into the genome of non-dividing cells, which is the unique feature of Lentiviruses as other Retroviruses can infect only dividing cells. The viral genome in the form of RNA is reverse transcribed when the virus enters the cell to produce DNA, which is then inserted into the genome at a random position by the viral integrase enzyme. The vector, now called a provirus, remains in the genome and is passed on to the progeny of the cell when it divides. The site of integration is unpredictable, which can pose a problem. The provirus can disturb the function of cellular genes and lead to activation of oncogenes promoting the development of cancer, which raises concerns for possible applications of lentiviruses in gene therapy. However, studies have shown that lentivirus vectors have a lower tendency to integrate in places that potentially cause cancer than gamma-retroviral vectors. More specifically, one study found that lentiviral vectors did not cause either an increase in tumor incidence or an earlier onset of tumors in a mouse strain with a much higher incidence of tumors. Moreover, clinical trials that utilized lentiviral vectors to deliver gene therapy for the treatment of HIV experienced no increase in mutagenic or oncologic events.

For safety reasons lentiviral vectors never carry the genes required for their replication. To produce a lentivirus, several plasmids are transfected into a so-called packaging cell line, commonly HEK 293. One or more plasmids, generally referred to as packaging plasmids, encode the virion proteins, such as the capsid and the reverse transcriptase. Another plasmid contains the genetic material to be delivered by the vector. It is transcribed to produce the single-stranded RNA viral genome and is marked by the presence of the ψ (psi) sequence. This sequence is used to package the genome into the virion.

Lentiviruses have the unique ability amongst retroviruses of being able to infect non-cycling cells. Vectors derived from lentiviruses have provided a huge advancement in technology and seemingly offer the means to achieve significant levels of gene transfer *in vivo*. As the particles are often pseudotyped with the envelope of the vesicular stomatitis virus (VSV), the vector can serve to introduce genes into a broad range of tissues and can be used *in vivo*. Furthermore, it has been demonstrated that *in vivo* expression is sustained for several months without detectable pathology. In the original lentivirus vectors described by Naldini et al (Naldini et al., 1996a) (Naldini et al., 1996b), the vector pCMVΔR8.2 supplies all but the HIV envelope in trans, the vector pMD.G is used to produce the VSV-G pseudotype, while the transgene (lac Z) is inserted into the plasmid pHR', which uses a lentiviral LTR, a splice donor and gag splice donor and acceptor associated with a rev responsive element. These three plasmids are used to transduce 293T cells and the supernatant assayed for p24 gag activity.

From these original experiments, new plasmids have been devised which have more versatile features. These include such plasmids as pLenti4/V5-DEST™, pLenti6/V5-DEST™, pLenti6.2/UbC/V5-DEST™ (Invitrogen Life Technologies Inc.) which utilize Gateway Technology™ (Invitrogen Life Technologies Inc.) in which recombination occurs

through the *attR1* and *attR2* and thus allows for rapid cloning Lentiviral systems for gene delivery are generally derived from the human immunodeficiency virus. The VDL utilizes a vector construct that employs a nonhomologous viral envelope (derived from the vesicular stomatitis virus), possess only three HIV genes, and separates the genes encoding the components necessary for viral packaging onto four different plasmids. These features, in conjunction with several other biosafety enhancements, prevent recombination of replication-competent HIV.

All lentivirus vectors produced in the VDL are produced in compliance with GLP standards. The production of a lentivirus virus begins with cloning the gene of interest (goi) into an entry plasmid of the Gateway Technology™ (Invitrogen). The entry plasmid containing your gene of interest is then recombined with one of the ViraPower™ Lentiviral Expression plasmids using Gateway Technology™ (Invitrogen). The ViraPower plasmids which lacks the gag/pol envelope genes and other accessory genes. The expression plasmid is cotransfected into 293FT cells with three supercoiled packaging plasmids (pLP1(gag/pol), pLP2 (rev) and pLP/VSV-G (VSV-G envelope) which supply helper functions and viral proteins in trans. At 48-72 hours post-transfection, the supernant (containing the viral particles) is harvested and clarified. At this point lentiviral vectors can be used to transduce the mammalian cell line of choice.

2.3 Electroporation

Electroporation involves exposure of cells in suspension to a pulsed electric field that causes transient formation of pores in the cell membrane, allowing exchange of macromolecules between the extracellular environment and the cytoplasm. Removal of the electric field results in spontaneous sealing of the pores, since the field strength and duration of the pulse are within the tolerable range for the cells. Parameters that affect the transfection efficiency of electroporation are discussed below.

2.3.1 Voltage

One of the most important factors affecting transfection efficiency by electroporation is the electric field strength across the cell which, at a critical point, leads to the localized breakdown of the cell membrane and formation of pores. The field strength is described by:

$$E = V/d$$

where E is the field strength, V is the voltage, and d is the distance between electrodes, there is a direct correlation between the field strength and voltage. Moreover, the field strength is also proportional to the product of the capacitor voltage and the cell diameter. Therefore the optimum voltage for different cell types varies and has to be determined empirically. Generally, efficient introduction of DNA into cells occurs at field strengths that give rise to 20-80% cell death. It is noteworthy that after electroporation many cells that exhibit trypan blue exclusion and adhere to the plate die and detach within 48 hours post-transfection and therefore counting the cells by trypan blue exclusion may provide an inaccurate measure of the number of viable cells.

2.3.2 Time constant

Most electroporations are performed using exponential decay waves that are generated by an instant surge of voltage followed by its exponential decline over a time period. This is defined as time constant and is a product of capacitance and the electroporation buffer resistance. Time constant is a useful parameter for ensuring reproducibility of the pulse. Some commercial electroporation apparatuses indicate the time constant after each pulse and provide the opportunity to compare different pulses and hence reproducibility of the electroporation.

To modify the time constant, the capacitance of the apparatus and/or the buffer resistance can be altered. Higher ionic strength of the buffer and shorter distance between the electrodes result in lower resistance. The resistance is however inversely proportional to the cross-sectional area of the path and therefore it is possible to manipulate the resistance by changing the volume of the electroporation buffer as well.

2.3.3 Buffer

The composition of the electroporation buffer determines its resistance and hence influences the time constant. In some cases growth media have also been used as the suspension medium. It is therefore essential to determine the best buffer/medium for each cell type empirically.

2.3.4 Temperature

There are different opinions with regards to the cell suspension temperature before, during, and after the electric pulse. Some reports indicate higher transfection frequencies at room temperature whereas others suggest incubation of cells in the presence of DNA on ice at least ten minutes before and after applying the pulse.

2.3.5 DNA

Increasing DNA concentration results in higher transfection frequency by electroporation. High transfection efficiencies have been reported with up to 80 ug/ml of DNA, Carrier nucleic acids have also been used to increase the transfection efficiency, but their nature appears to play an important role. For instance salmon sperm DNA increases transfection efficiencies of some human cell types whereas yeast tRNA results in lower transfection frequencies.

2.3.6 Cells

Like other transfection methods the type of cells and the cell cycle phase at the time of electroporation are important factors in determining the efficiency of transfection by electroporation. In most cases best results are obtained with 1×10^6 to 2×10^7 cells/ml at exponential growth phase.

2.4 Staining of cells for expression of β-galactosidase
Factors that influence the efficiency of a gene delivery system have to be tested empirically to obtain the best conditions for a particular cell line. For this purpose, use can be made of reporter genes, including the genes that code for β-galactosidase, luciferase, and chloramphenicol acetyl transferase (CAT). The reporter gene can be transfected into cells and

its expression analysed by a biochemical assay and/or histochemical staining 24-72 hours post-transfection. Because there tends to be a correlation between transient expression of the reporter gene and stable transformation of cells, this method can be used to determine the optimum conditions for both transient and stable transfections.

2.5 Rescue of episomal plasmids

This method was originally described by Hilt for the isolation of viral DNA from mouse cells. The episomal Epstein-Barr virus (EBV) vectors such as the pREP series of expression vectors (Invitrogen) can be rescued from transfected cells and used to transform *E. coli* cells for their propagation.

3 Microcell-mediated chromosome transfers

Cell-cell fusion of somatic cells is a simple way of transferring genetic material from one cell to another, where all the chromosomes of the participating cells are engulfed within a single cell membrane. A more refined method of transferring genetic information is the method of microcell-mediated chromosome transfer (MMCT), where only one chromosome, or a small group of chromosomes, is transferred to the recipient cell.

The MMCT technique has been a powerful tool in a variety of studies involving the transfer of human chromosomes to rodent cells and the generation of monochromosomal human-rodent cell hybrids. However, because rodent cell hybrids lose the human chromosomes at high frequencies, one of the first considerations is to have the means for selecting the cells that have received and retained the desired chromosome. Naturally occurring markers such as dihydrofolate reductase, thymidine kinase, and hypoxanthine-guanine phosphoribosyl transferase, on chromosomes 5, 17, and X, respectively, can be used to isolate human-rodent hybrids that carry these chromosomes. However, for the majority of the human chromosomes exogenous markers have to be used to tag the chromosomes before their transfer into the recipient rodent cells. The two most widely used markers for this purpose have been the *nco* gene, which confers resistance to neomycin, and the bacterial xanthine-guanine phosphoribosyl transferase (gpt) gene, which enables mammalian cells carrying this gene to utilize xanthine rather than hypoxanthine in the salvage pathway of purine biosynthesis. Methods such as calcium phosphate co-precipitation and retroviral transduction have been used to transfer these markers into human cells and to generate monochromosomal hybrids.

Microcell-mediated chromosome transfer has been particularly useful in the mapping of DNA repair genes. In these studies, complementing DNA repair genes have been assigned to particular chromosomes by individually transferring human chromosomes into DNA repair defective cell lines and identifying the chromosomes that restore proficient DNA repair. This technique has also been used in other studies such as chromosomal localization of tumour suppressor genes, gene regulation, manipulation of chromosomal alleles and introduction of human minichromosomes into mouse embryonal stem cells. The donor cells in MMCT experiments are usually rodent cells carrying a single human chromosome, tagged with a selectable marker such as *gpt, neo,* or *hyg,* and are called mouse-human monochromosomal hybrids. Several groups have constructed monochromosomal hybrid panels that can be obtained directly from the laboratories that constructed them, purchased from commercial centres or from public resource institutes, such as Coriell Cell Repositories (http://locus.umdnj.edu/nigms/hybrids/sumintro.html) and the UK MRC Human Genome

Mapping Project Resource Centre (http://www.hgmp.mrc. ac. uk/Research/). It must be noted that in some cases in addition to the selected intact human chromosome, a monochromosomal hybrid may have retained a small fragment(s) of another human chromosome and therefore careful characterization of these hybrids is essential before their use in any chromosome transfer experiment.

The procedure of MMCT involves five main stages, which are illustrated in *Figure 1* and described below.
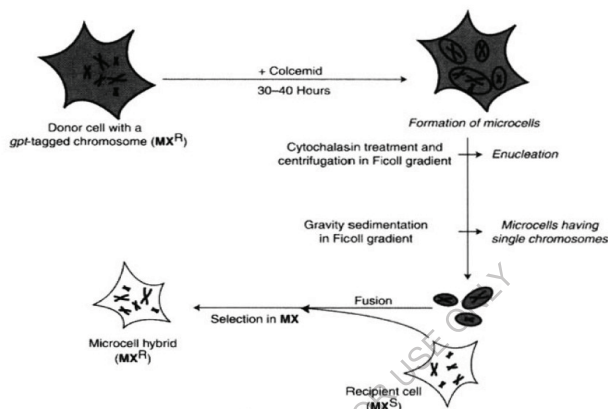
Figure 1. Microcell-mediated chromosome transfer. In this method a single chromosome that is tagged with a dominant marker (such as the bacterial guanine phosphoribosyl transferase *(gpt)* gene) is encapsulated into a microcell by colcemid-induced micronucleation of its donor cell. The microcell is then extruded from the micronucleate cell by centrifugation in a discontinuous Ficoll gradient containing cytochalasin B and then size fractionated in a continuous Ficoll gradient. Fusion of the microcell to a recipient cell and growth in MX medium (medium containing mycophenolic acid and xanthine) gives rise to colonies of microcell hybrids that contain the tagged chromosome from the donor cell. MX = culture medium containing mycophenolic acid and xanthine. MXR = resistant to MX. MXS = sensitive to MX.

3.1 Formation of micronuclei

Micronucleation describes the process of formation of micronuclei and is induced by treating donor cells with a mitotic inhibitor such as colcemid. Treatment of the cells with sublethal doses of colcemid results in disruption of microtubule formation during cell division followed by the formation of micronuclei when the cells enter interphase. Each micronucleus contains one or a few chromosomes within a membrane.

The appropriate dose of colcemid has to be determined empirically. In general, micronucleation can be induced in rodent cells with lower doses (0.01-0.10 µg/ml) of colcemid than in human cells, which need higher concentrations (10-20 ng/ml) of this drug. In the MMCT, micronucleation is achieved by prolonged incubation (30-40 hours) of mouse human monochromosomal hybrid cells, as donors of the human chromosome, in medium

containing 0.02 μg/ml of colcemid. Another method has been described in which the donor cells are treated with colcemid for a shorter period of time (8-16 hours) followed by incubation for 4-6 hours at 37°C in medium containing cytochalasin B, which is a mitotic inhibitor and induces nuclear extrusion.

3.2 Enucleation

When micronucleate cells are treated with relatively high concentrations of cytochalasin B (10 μg/ml) the micronuclei can be extruded in two ways: as monolayer cells or as cells in suspension.

3.2.1 Enucleation of monolayer cells

Micronucleate cells can be enucleated while they are still attached to the surface of either a tissue culture flask or to sterile pieces of plastic cut from tissue culture plates in the shape of disks or bullets. If the cells are attached to a tissue culture flask, the flask is filled with serum-free medium containing 10 μg/ml cytochalasin B and then centrifuged. If, Conversely, micronucleate cells have been generated on disks or bullets, they are enucleated by transferring the disks, cell side down, to 50 ml centrifuge tubes containing serum-free medium supplemented with 10 ug/ml of cytochalasin B and centrifuged at high speed. The *g* force of the centrifugation, of course, must not exceed the tolerable limit for the tissue culture flask or the pre-cut plastic pieces.

3.2.2 Enucleation in suspension

Enucleation is achieved by first harvesting the micronucleate cells by trypsinization and then centrifuging them in a discontinuous Ficoll gradient in the presence of cytochalasin B.

3.3 Purification of microcells

The end-result of enucleation of micronucleate cells is the release of microcells as compartments containing single, or just a few chromosomes, with a thin rim of cytoplasm encircled by a cell membrane. Microcells can be purified on the basis of their size either by unit gravity sedimentation or by filtration through polycarbonate nitres with pore size of 5 μm or 8 μm. alternatively; the crude microcell preparation is transferred into tissue culture flasks followed by incubation at 37°C for several hours. In this period of incubation, which varies for different donor cell types, the contaminating cells that are intact adhere to the surface of the tissue culture flasks and the microcclls, which are non-adhering, remain floating in the medium and can be collected.

3.4 Fusion of microcells to recipient cells

There are two main methods for the fusion of microcells to the recipient cells. One method uses polyethylene glycol (PEG) in combination with phytohaemagglutinin-P (PHA-P). The role of PHA-P in this procedure is to increase agglutination of the microcells to the surface of the recipient cells and hence enhance the fusion efficiency. The optimum duration of PEG treatment has to be determined empirically for each cell type. An alternative method uses inactivated Sendai virus for fusing the microcells to the recipient cells, but the former method is more widely used, owing to its efficiency and simplicity.

3.5 Selection of microcell hybrids

In this step the monochromosomal hybrids are selected by growing the fusion products in medium containing an appropriate selective agent. If the human chromosomes are tagged with the neo or *hyg* genes, neomycin or hygromycin are used to select the monochromosomal hybrids respectively. If, Conversely, the human chromosomes are tagged with the gpt gene, the selection is performed by growing the cells in MX medium, containing mycophenolic acid and xanthine. The *gpt* gene is the bacterial analogue of the mammalian *hprt* gene in the purine biosynthesis pathway. The product of this gene utilizes xanthine instead of hypoxanthine, which is the substrate for the *hprt* gene product. Selection in MX medium is therefore based on blocking the endogenous pathway of purine biosynthesis by mycophenolic acid and supplementing the medium with xanthine. Therefore, only the cells containing the *gpt* gene product can utilize the xanthine component of the selection medium to bypass the inhibitory effect of mycophenolic acid and survive.
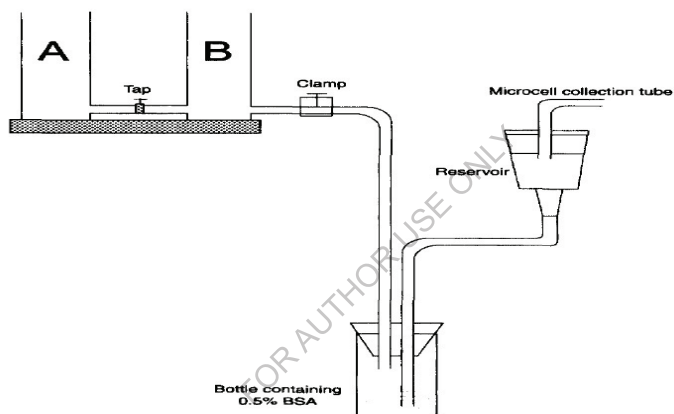
Figure 2. Gradient system for size fractionation of microcells. This is a schematic diagram of a system for purification of microcells by unit gravity sedimentation in a continuous Ficoll gradient. The gradient maker is connected to a 10 ml bottle via a tube that is sealed with a clamp. Thus the clamp can control the flow of solution through the system. The bottle is also connected to the bottom of a reservoir, which can be the barrel of a 50 ml syringe. Two magnetic stirrers, one in chamber B of the gradient maker and one in the bottle, are used to mix the solutions gently. The components of this system have to be sterile and assembled on *a* non-vibrating bench. To prevent contamination, it is necessary to cover the gradient maker chambers and the reservoir with pre-sterilized aluminium foil throughout the process, before and immediately after the addition of solutions. This and supplementing the culture medium with amphotericin B after the fusion step helps prevent yeast and fungal infection of the cells.

4 Irradiation fusion gene transfer

The technique of irradiation fusion gene transfer (IFGT) allows the generation of a varied array of hybrids that can be used in investigations such as gene mapping and/or

positional cloning of genes. Figure 3 is a schematic illustration of the IFGT technique for reducing the amount of human DNA in somatic cell hybrids.
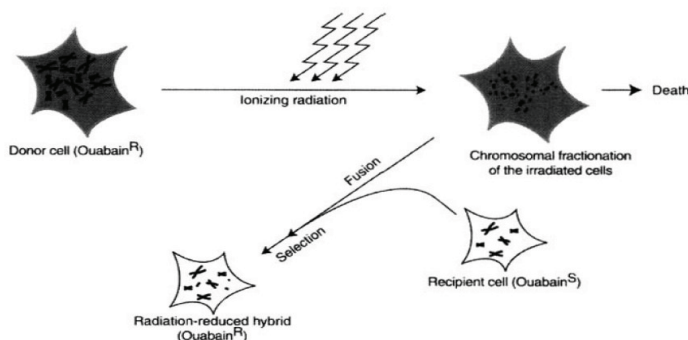
Figure 3. Irradiation fusion gene transfer. Chromosomes of donor cells that carry a selectable marker are fragmented by exposure of the cells to high doses of ionizing radiation. The irradiated cells are then rescued by fusing them to a recipient cell line that is sensitive to the selective marker. The resulting hybrids that retain fragments of the donor cell chromosomes are isolated by selection in medium containing the selective marker. OuabainR = resistant to ouabain. OuabainS - sensitive to ouabain.

4.1 Use in mapping genes

The IFGT technique was first introduced by Goss and Harris (54). They based their work on two observations:

a. Ionizing radiation breaks chromosomes into smaller fragments with sizes that are proportional to the radiation dose. Therefore, the closer two genes are together the more likely they are to segregate within a segment of DNA after treatment with ionizing radiation.
b. Fragments of foreign DNA can, under certain conditions, be introduced into mammalian cells where they can replicate and express their genes.

In their study Goss and Harris determined the linkage between four X chromosome genes and their order. These genes were those coding for the enzymes hypoxanthine guanine phosphoribosyl transferase (HPRT), phosphoglycerate kinase (PGK), glucose-6-phosphate dehydrogenase (G6PD), and a-galactosidase (a-gal). Irradiated lymphocytes were fused to a hamster cell line lacking HPRT activity. Unfused lymphocytes were removed by washing and the hybrids were grown under HAT selection (medium containing hypoxanthine, aminopterin, and thymidine). Therefore, only the recipients of the humant HPRT gene could survive. It was shown that both the gene order and distance between the genes could be determined by this technique.

IFGT was also used to map 14 DNA probes from a region of human chromosome 21q spanning 21 Mb. These experiments started with a Chinese hamster/human somatic cell hybrid containing a single copy of human chromosome 21q and very little other human

chromosomal material. The hamster/human hybrid cells were irradiated with 80 Gy X-rays. The lethally irradiated cells were fused to hamster cells lacking the HPRT gene, and the hybrids were selected in HAT medium. In this selection only those hybrids which had received the hamster HPRT gene from the irradiated cells could survive. Therefore, there was no direct selection for human sequences. The presence of the markers in the radiation hybrids was confirmed by Southern blot hybridization analysis and it was found that each of the 14 chromosome 21 markers was retained in 30-60% of the radiation hybrids. Statistical analysis on the results was then used to construct a map of the markers on chromosome 21. These mapping data were in agreement with those obtained from pulse-field gel electrophoresis studies, indicating that it is possible to determine the distance between different markers, and their order on the chromosome, by estimating the frequency of breakage between them. Radiation hybrid panels have become very useful tools in mapping human genes because they can be screened by polymorphic as well as non-polymorphic markers, which are uninformative in linkage mapping. One such panel is the GeneBridge4 radiation hybrid mapping panel which is available from the UK MRC Human Genome Mapping Project Resource Centre, UK MRC HGMP-RC (http://www.hgmp.mrc.ac.uk/Research/). This panel consists of 86 clones of the whole human genome and can be used for ordering genetic markers and estimating their distances. After obtaining the panel, the investigator screens this panel with the marker of interest and submits the data to the UK MRC HGMP-RC, where they are statistically analysed and the results returned.

4.2 Use in positional cloning

The most important factor in the positional cloning of a gene is the identification or isolation of markers that are tightly linked to that gene. One possible way to achieve this is to use the method of IFGT to construct a panel of radiation reduced hybrids. If, for example, the aim is to clone a human gene, for which no tightly linked marker has been identified, a hybrid is generated that contains the smallest possible chromosome fragment carrying the gene of interest. In this case the best donor cell is a human-rodent monochromosomal hybrid or a hybrid that contains only a segment of the human chromosome carrying the gene. The generated radiation reduced hybrids can then be analysed using techniques such as polymerase chain reaction (PCR) or Southern blot hybridization for the presence of known markers in the critical region. This can lead to the identification of a marker that is closely linked to the gene. Moreover, new sequence tagged site (STS) markers can be generated from the hybrid with the least amount of human DNA by the method of inter-Alu-repeat sequence PCR (IRS-PCR), which amplifies sequences between the Alu-repeat elements. The new STS markers are then analysed for their linkage to the gene and for isolation of yeast artificial chromosomes (YACs) that serve as the primary tools for the next stage of positional cloning.

A point to consider in the generation of radiation reduced hybrids for positional cloning by IFGT is that because the selectable marker on the human chromosome is likely to be too far away from the gene, direct selection for the human chromosome cannot be applied. Therefore it is necessary to generate donor monochromosomal hybrid cells with a selectable marker (e.g, ouabain resistance) on the non-human chromosomes, or use recipient cells that are defective in a gene such as HPRT. Thus, selection of the radiation reduced hybrids is achieved by growing the cells in ouabain or HAT medium. This, therefore, limits the use of IFGT in positional cloning of a gene to instances where the gene has phenotypic effects in the resulting hybrids or where flanking markers have been identified. An example of successful use of the IFGT technique in positional cloning of a gene is the identification of ku80 as the mutant gene in the DNA repair deficient xrs cells.

4.3 Irradiation doses

The relationship between the dose of irradiation and the size or number of human DNA fragments retained in the recipient cells has been studied by using a hamster/human somatic cell hybrid containing human chromosomes 3 and X as the source of human DNA fragments. These cells were irradiated with a lethal dose of 25-250 Gy y-rays. The irradiated cells were rescued by fusing them to a thymidine kinase' (TK-) hamster cell line followed by selection of the radiation hybrids in HAT medium, allowing only the survival of hybrids receiving the hamster Tk gene. Alu-PCR analysis showed the presence of unselected human DNA sequences in the isolated hybrids. The presence or absence of markers specific to chromosome 3 or X in the hybrids was also tested. This analysis as well as fluorescence in situ hybridization (FISH) studies on the radiation hybrids suggested that the number of human DNA fragments in these hybrids was independent of the irradiation dose. However, the sizes of the fragments were dose-dependent. By increasing the irradiation dose from 50 to 250 Gy a five- to tenfold reduction in the sizes of the largest fragments was observed. Also human marker retention frequencies declined from 0.27 to 0.03. The Xq27-Xq28 region was used as a model to estimate the size range of the retained fragments in the radiation hybrids. 40% of the hybrids generated following 50 Gy y-rays retained fragments in the range of 3-30 Mb, 10% retained the whole chromosome arm, and the remaining 50% retained fragments of less than 2-3 Mb. The retention of 3 Mb or larger fragments decreased to less than 6% in hybrids generated at the higher dose of 250 Gy.

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Chapter 11
Stem Cells

1 Stem Cell

Stem cells are a class of undifferentiated cells that are able to differentiate into specialized cell types found in most, if not all, multi-cellular organisms. Research in the stem cell field grew out of findings by Canadian scientists Ernest A. Mc Culloch and James E. Commonly, stem cells come from two main sources: Embryos formed during the blastocyst phase of embryological development (embryonic stem cells) and Adult tissue (adult stem cells). Both types are generally characterized by their potency, or potential to differentiate into different cell types (such as skin, muscle, bone, etc.). In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. Stem cells can now be grown and transformed into specialized cells with characteristics consistent with cells of various tissues such as muscles or nerves through cell culture. Highly plastic adult stem cells from a variety of sources, including umbilical cord blood and bone marrow, are routinely used in medical therapies. Embryonic cell lines and autologous embryonic stem cells generated through therapeutic cloning have also been proposed as promising candidates for future therapies.

2 Properties

The classical definition of a stem cell requires that it possess two properties:

1. ***Self-renewal*** - the ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
2. ***Potency*** - the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotent- to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred to as stem cells.

3 Potency

- Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta.
- Totipotent - the ability to differentiate into all possible cell types. Examples are the zygote formed at egg fertilization and the first few cells that result from the division of the zygote.
- Pluripotent - the ability to differentiate into most cell types. Examples include embryonic stem cells and cells that are derived from the mesoderm, endoderm, and ectoderm germ layers that are formed in the beginning stages of embryonic stem cell differentiation.
- Multipotent - the ability to differentiate into a closely related family of cells. Examples include hematopoietic (adult) stem cells that can become red and white blood cells or platelets.
- Oligopotent - the ability to differentiate into a few cells. Examples include (adult) lymphoid or myeloid stem cells.

- Unipotent - the ability to only produce cells of their own type, but have the property of self-renewal required to be labeled a stem cell. Examples include (adult) muscle stem cells.

4 Basic principles for identification and purification of stem cells

Although there is not complete agreement among scientists of how to identify stem cells, most tests are based on making sure that stem cells are undifferentiated and capable of self-renewal. Tests are often conducted in the laboratory to check for these properties.

One way to identify stem cells in a lab, and the standard procedure for testing bone marrow or hematopoietic stem cell (HSC), is by transplanting one cell to save an individual without HSCs. If the stem cell produces new blood and immune cells, it demonstrates its potency.

Clonogenic assays (a laboratory procedure) can also be used *in vitro* to test whether single cells can differentiate and self-renew. Researchers may also inspect cells under a microscope to see if they are healthy and undifferentiated or they may examine chromosomes.

To test whether human embryonic stem cells are pluripotent, scientists allow the cells to differentiate spontaneously in cell culture, manipulate the cells so they will differentiate to form specific cell types, or inject the cells into an immunosuppressed mouse to test for the formation of a teratoma (a benign tumor containing a mixture of differentiated cells).

Epithelial tissues, such as the epidermis and the lining of the gut, share a common feature in that they are constantly shedding cells from their outer surface. This constant cell loss is compensated by continual replacement through cell proliferation in a highly regulated process. In humans the entire outer layer of the skin is shed daily, while the entire epithelial lining of the mouse gut is replaced every three to four days. As this process continues throughout life it has been argued that this is proof of the existence of long-lived stem cells. The definition of such a stem cell is that it lacks certain tissue-specific differentiation markers, remains in the tissue throughout life, retaining proliferative capacity, and gives rise to daughters, some of which generate differentiated cells and others which are themselves stem cells. The cells should also be capable of regenerating the tissue after injury. It has been demonstrated in the epidermis that the proliferative compartment consists not only of stem cells and post-mitotic cells, but also of a transit amplifying population. Transit amplifying cells are produced initially by the division of stem cells and, although they have a high proliferative capacity, their lifespan is limited and eventually all the cells will differentiate and be shed from the tissue. When approaching the question of whether or not a particular tissue contains a discrete stem cell population it is necessary to establish whether there is true proliferative heterogeneity within the tissue. There are three possibilities: first, all the cells may have equal proliferative potential, secondly, only a subpopulation of non-differentiated cells may divide or thirdly, as in the skin, there are two types of proliferative cell, stem cells and transit amplifying cells. The latter may be represented by a continuous distribution of proliferative capacity, from unlimited to a single division only. Alternatively there may well be two clear proliferative subpopulations that can be distinguished by criteria such as the morphology of colonies produced in cell culture.

4.1 Assessment of proliferative heterogeneity

In the epidermis it has been shown that there is proliferative heterogeneity within the keratinocyte population. Barrandon and Green showed that there are three types of colony formed when these cells are placed in culture, in high calcium medium, in the presence of a 3T3 feeder layer. The three colony types formed are called paraclones, meroclones, and holoclones, and these classifications are based on the type of progeny produced by the colonies when they are passaged into fresh dishes (Figure 1). Paraclones form small, irregular colonies, composed almost entirely of differentiated cells. Colonies produced by holoclones are large and round, consisting mostly of small cells, surrounding a central region of stratified differentiated cells. Meroclones produce colonies which do not have a smooth outline and are smaller than those produced by holoclones. It is believed that the stem cells produce holoclones and transit amplifying cells, paraclones. The origin of meroclones in this definition is not clear, although it has been speculated that they are produced by stem, cells that generate transit amplifying cells at a higher rate than those producing holoclones. Epithelial cells from other tissues, including prostate, also show this heterogeneity in clonal growth properties and this type of experimental approach could well be of use in stem cell studies in the breast, gut epithelium, and liver. As for epidermal keratinocytes, three colony types are formed: small abortive clones, large, round, fast growing colonies made up almost exclusively of small cells, and an intermediate colony type which may grow rapidly but contains larger differentiated cells and is irregular in shape. Cloning is a useful tool for the assessment of proliferative capacity and differentiation state of different colonies. If the cell type can be cloned from single cells it is possible to avoid ring cloning altogether and grow single colonies in 32 mm dishes.
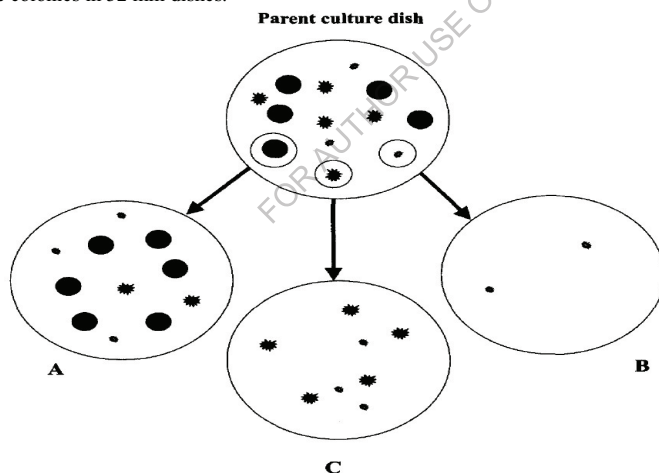
Figure 1. When keratinocytes are cultured at low density three types of colony are seen in the parent culture dish. Subcloning of the largest type of colony (A) yields many large colonies, along with several small and some abortive colonies. Subcloning a small colony (C) produces only small and abortive colonies, whereas an abortive colony (B) will produce no colonies. Large colony-forming cells behave like stem cells whereas small colony-forming cells behave like transit amplifying cells.

5 Embryonic stem cells

Embryonic stem cells are derived from a four- or five-day-old human embryo that is in the blastocyst phase of development. The embryos are usually extras that have been created in IVF (*in vitro* fertilization) clinics where several eggs are fertilized in a test tube, but only one is implanted into a woman.

Sexual reproduction begins when a male's sperm fertilizes a female's ovum (egg) to form a single cell called a zygote. The single zygote cell then begins a series of divisions, forming 2, 4, 8, 16 cells, etc. After four to six days - before implantation in the uterus - this mass of cells is called a blastocyst. The blastocyst consists of an inner cell mass (embryoblast) and an outer cell mass (trophoblast). The outer cell mass becomes part of the placenta, and the inner cell mass is the group of cells that will differentiate to become all the structures of an adult organism. This latter mass is the source of embryonic stem cells - totipotent cells (cells with total potential to develop into any cell in the body).
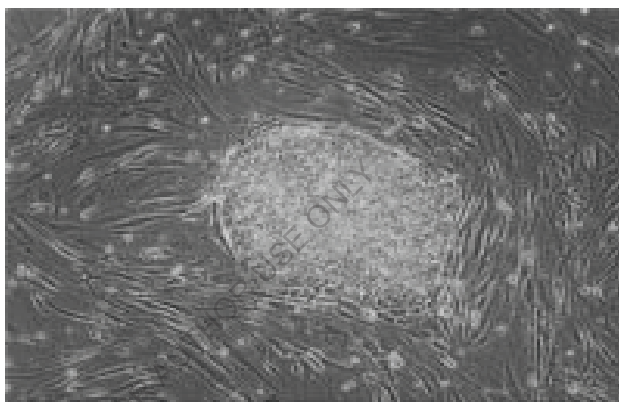
Figure 2. Human embryonic stem cell colony

In a normal pregnancy, the blastocyst stage continues until implantation of the embryo in the uterus, at which point the embryo is referred to as a fetus. They do not contribute to the extra-embryonic membranes or the placenta. This usually occurs by the end of the 10th week of gestation after all major organs of the body have been created. However, when extracting embryonic stem cells, the blastocyst stage signals when to isolate stem cells by placing the "inner cell mass" of the blastocyst into a culture dish containing a nutrient-rich broth. Lacking the necessary stimulation to differentiate, they begin to divide and replicate while maintaining their ability to become any cell type in the human body. Eventually, these undifferentiated cells can be stimulated to create specialized cells.

Nearly all research to date has taken place using mouse embryonic stem cells (mES) or human embryonic stem cells (hES) (Figure 2). Both have the essential stem cell characteristics, yet they require very different environments to maintain an undifferentiated state. Mouse ES cells are grown on a layer of gelatin and require the presence of Leukemia Inhibitory Factor (LIF). Human ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic Fibroblast Growth Factor (bFGF or FGF-2). Without optimal culture conditions or genetic manipulation, embryonic stem cells will rapidly differentiate.

A human embryonic stem cell is also defined by the presence of several transcription factors and cell surface proteins. The transcription factors Oct-4, Nanog and Sox2 form the core regulatory network that ensures the suppression of genes that lead to differentiation and the maintenance of pluripotency. The cell surface antigens most commonly used to identify hES cells are the glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens Tra-1-60 and Tra-1-81. The molecular definition of a stem cell includes many more proteins and continues to be a topic of research.

After nearly ten years of research, there are no approved treatments using embryonic stem cells. The first human trial was approved by the US Food & Drug Administration in January 2009. ES cells, being pluripotent cells, require specific signals for correct differentiation - if injected directly into another body; ES cells will differentiate into many different types of cells, causing a teratoma. Differentiating ES cells into usable cells while avoiding transplant rejection are just a few of the hurdles that embryonic stem cell researchers still face. Many nations currently have moratoria on either ES cell research or the production of new ES cell lines. Because of their combined abilities of unlimited expansion and pluripotency, embryonic stem cells remain a theoretically potential source for regenerative medicine and tissue replacement after injury or disease.

Additionally, under defined conditions, embryonic stem cells are capable of propagating themselves indefinitely. This allows embryonic stem cells to be used as useful tools for both research and regenerative medicine, because they can produce limitless numbers of themselves for continued research or clinical use.

Because of their plasticity and potentially unlimited capacity for self-renewal, ES cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. However Diseases treated by these non-embryonic stem cells include a number of blood and immune-system related genetic diseases, cancers, and disorders; juvenile diabetes; Parkinson's; blindness and spinal cord injuries.

5.1 Differentiation of embryonic stem cells
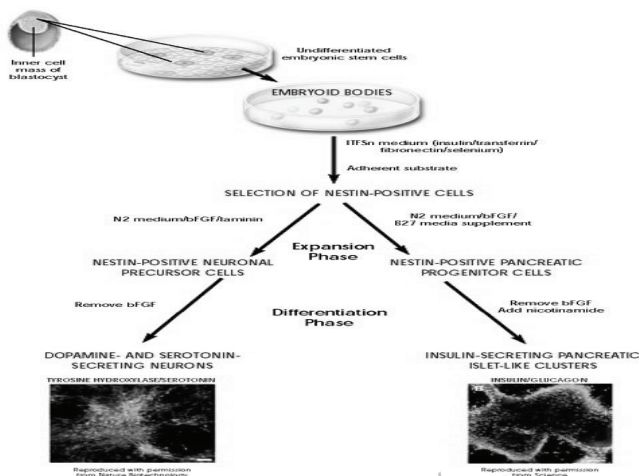
Figure 3. Directed differentiation of mouse embryonic stem cells

Since the embryonic stem cells in culture are grown under certain conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, it is not an efficient way to produce cultures of specific cell types.

So, to generate cultures of specific types of differentiated cells-heart muscle cells, blood cells, or nerve cells, for example-scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some specific cell types (Figure 3).

5.2 Isolation and *in vitro* culture

In 1964, researchers isolated a single type of cell from a teratocarcinoma, a form of cancer that replicated and grew in cell culture as a stem cell. Subsequently, researchers isolated a primordial embryonic germ cell (EG cell) that, after replicating and growing in cell culture as a stem cell, was capable of developing into different cell types.

In 1981, embryonic stem cells (ES cells) were first derived from mouse embryos by Martin Evans and Matthew Kaufman at the Department of Genetics, University of Cambridge and independently by Gail R. Martin. Martin is credited with coining the term "Embryonic Stem Cell".

If scientists can reliably direct the differentiation of embryonic stem cells into specific cell types, they may be able to use the resulting, differentiated cells to treat certain diseases at some point in the future. Diseases that might be treated by transplanting cells generated from human embryonic stem cells include Parkinson's disease, diabetes, traumatic spinal cord injury, Purkinje cell degeneration, Duchenne's muscular dystrophy, heart disease, and vision and hearing loss.

5.3 Contamination by reagents used in cell culture

The online edition of *Nature Medicine* published a study on January 24, 2005 which stated that the human embryonic stem cells available for federally funded research are contaminated with non-human molecules from the culture medium used to grow the cells. It is a common technique to use mouse cells and other animal cells to maintain the pluripotency of actively dividing stem cells. The problem was discovered when non-human sialic acid in the growth media was found to compromise the potential uses of the embryonic stem cells in humans.

After more than 6 months of undifferentiated proliferation, these cells demonstrated the potential to form derivatives of all three embryonic germ layers both *in vitro* and in teratomas. These properties were also successfully maintained (for more than 30 passages) with the established stem cell lines.

5.4 Reducing donor-host rejection

There is also ongoing research to reduce the potential for rejection of the differentiated cells derived from ES cells once researchers are capable of creating an approved therapy from ES cell research. One of the possibilities to prevent rejection is by creating embryonic stem cells that are genetically identical to the patient via therapeutic cloning.

An alternative solution for rejection by the patient to therapies derived from non-cloned ES cells is to derive many well-characterized ES cell lines from different genetic backgrounds and use the cell line that is most similar to the patient; treatment can then be tailored to the patient, minimizing the risk of rejection.

5.5 Therapeutic application

On January 23, 2009, Phase I clinical trials for transplantation of a human-ES-derived cell population into spinal cord injured individuals received approval from the US Food & Drug Administration (FDA), marking it the world's first human ES cell human trial. The study leading to this scientific advancement was conducted by Hans Keirstead and colleagues at the University of California. The results of this experiment suggested an improvement in locomotor recovery in spinal cord-injured rats after a 7-day delayed transplantation of human ES cells that were pushed towards an oligodendrocytic lineage.

Use of human embryonic stem cells as models for human genetic disorders

In recent years there have been several reports regarding the potential use of human embryonic stem cells as models for human genetic diseases. This issue is especially important due to the species-specific nature of many genetic disorders. The relative inaccessibility of

human primary tissue for research is another major hindrance. Several new studies have started to address this issue. This has been done either by genetically manipulating the cells, or more recently by deriving diseased cell lines identified by prenatal genetic diagnosis (PGD). This approach may very well prove invaluable at studying disorders such as Fragile-X- syndrome, Cystic fibrosis and other genetic maladies that have no reliable model system.

The techniques of prenatal diagnosis testing methods to determine genetic and chromosomal disorders are now used by many pregnant women and prospective parents, especially those couples with a history of genetic abnormalities or where the woman is over the age of 35, when the risk of genetically-related disorders is higher. In addition, by allowing parents to select an embryo without genetic disorders, they have the potential of saving the lives of siblings that already had similar disorders and diseases using cells from the disease free offspring.

5.6 Embryonic stem cell trial approved by the FDA

In the summer of 2009, the first clinical trial using embryonic stem cells in humans was approved by the US Food & Drug Administration (FDA). A biotech company called the Geron Corporation will be conducting the trial. The study will involve the injection of neural stem cells into paraplegics who have suffered a recent spinal cord injury. About eight to ten paraplegics who have had their injuries no longer than two weeks before the trial begins, will be selected to take part in the trial, because the cells must be injected before scar tissue is able to form. However, the researchers are emphasizing that the injections are not expected to fully cure the patients and restore all mobility. Based on the results of the mice trials, researchers say restoration of myelin sheathes, and an increase in mobility is probable. This first trial is mainly testing the safety of these procedures and if everything goes well, it could lead to future studies that involve people with more severe disabilities.

6 Fetal stem cells

Fetal stem cells are primitive cell types found in the organs of fetuses [17]. The classification of fetal stem cells remains unclear and this type of stem cell is currently often grouped into an adult stem cell. However, a more clear distinction between the two cell types appears necessary.

7 Adult Stem Cells

Adult or somatic stem cells exist throughout the body after embryonic development and are found inside of different types of tissue. These stem cells have been found in tissues such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and the liver. They remain in a quiescent or non-dividing state for years until activated by disease or tissue injury. Adult stem cells can divide or self-renew indefinitely, enabling them to generate a range of cell types from the originating organ or even regenerates the entire original organ. It is generally thought that adult stem cells are limited in their ability to differentiate based on their tissue of origin, but there is some evidence to suggest that they can differentiate to become other cell types.

Pluripotent adult stem cells are rare and generally small in number but can be found in a number of tissues including umbilical cord blood. A great deal of adult stem cell research has focused on clarifying their capacity to divide or self-renew indefinitely and their

differentiation potential. In mice, pluripotent stem cells are directly generated from adult fibroblast cultures. Unfortunately, many mice don't live long with stem cell organs. Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin.

Adult stem cell treatments have been successfully used for many years to treat leukemia and related bone/blood cancers through bone marrow transplants. Adult stem cells are also used in veterinary medicine to treat tendon and ligament injuries in horses.

The use of adult stem cells in research and therapy is not as controversial as embryonic stem cells, because the production of adult stem cells does not require the destruction of an embryo. Additionally, because in some instances adult stem cells can be obtained from the intended recipient, (an autograft) the risk of rejection is essentially non-existent in these situations. Consequently, more US government funding is being provided for adult stem cell research.

7.1 Sources

Pluripotent stem cells, i.e., cells that can give rise to any fetal or adult cell type, can be found in a number of tissues, including umbilical cord blood. Using genetic reprogramming, pluripotent stem cells equivalent to embryonic stem cells have been derived from human adult skin tissue. Other adult stem cells are multipotent, meaning they are restricted in the types of cell they can become, and are generally referred to by their tissue origin (such as mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, etc.). A great deal of adult stem cell research has focused on investigating their capacity to divide or self-renew indefinitely, and their potential for differentiation. In mice, pluripotent stem cells can be directly generated from adult fibroblast cultures.

Amniotic

Multipotent stem cells are also found in amniotic fluid. These stem cells are very active, expand extensively without feeders and are not tumorogenic. Amniotic stem cells are multipotent and can differentiate in cells of adipogenic, osteogenic, myogenic, endothelial, hepatic and neuronal lines.

Induced Pleuripotent

These are not adult stem cells, but rather reprogrammed cells (e.g epithelial cells) given pluripotent capabilities. Using genetic reprogramming with protein transcription factors, pluripotent stem cells equivalent to embryonic stem cells have been derived from human adult skin tissue. Shinya Yamanaka and his colleagues used the transcription factors Oct3/4, Sox2, c-Myc, and Klf4 in their experiments on cells from human faces. Junying Yu and their colleagues used a different set of factors, Oct4, Sox2, Nanog and Lin28, and carried out their experiments using cells from human foreskin.

As a result of the success of these experiments, Ian Wilmut, who helped create the first cloned animal Dolly, has announced that he will abandon nuclear transfer as an avenue of research.

7.2 Signaling pathways

Adult stem cell research has been focused on uncovering the general molecular mechanisms that control their self-renewal and differentiation.

1. Bmi-1

The Transcriptional repressor Bmi-1s is one of the Polycomb-group proteins that was discovered as a common oncogene activated in lymphoma and later shown to specifically regulate HSCs. The role of Bmi-1 has also been illustrated in neural stem cells.

2. Notch

The Notch pathway has been known to developmental biologists for decades. Its role in control of stem cell proliferation has now been demonstrated for several cell types including haematopoietic, neural and mammary stem cells.

3. Wnt

These developmental pathways are also strongly implicated as stem cell regulators.

7.3 Lineage

To ensure self-renewal, stem cells undergo two types of cell division. Symmetric division gives rise to two identical daughter cells both endowed with stem cell properties. Asymmetric division, Conversely, produces only one stem cell and a progenitor cell with limited self-renewal potential. Progenitors can go through several rounds of cell division before terminally differentiating into a mature cell. It is possible that the molecular distinction between symmetric and asymmetric divisions lies in differential segregation of cell membrane proteins (such as receptors) between the daughter cells.

An alternative theory is that stem cells remain undifferentiated due to environmental cues in their particular niche. Stem cells differentiate when they leave that niche or no longer receive those signals. Studies in *Drosophila* germarium have identified the signals dpp and adherens junctions that prevent germarium stem cells from differentiating.

The signals that lead to reprogramming of cells to an embryonic-like state are also being investigated. These signal pathways include several transcription factors including the oncogene c-Myc. Initial studies indicate that transformation of mice cells with a combination of these anti-differentiation signals can reverse differentiation and may allow adult cells to become pluripotent. However, the need to transform these cells with an oncogene may prevent the use of this approach in therapy.

7.4 Multidrug resistance

Adult stem cells express transporters of the ATP-binding cassette family that actively pump a diversity of organic molecules out of the cell. Many pharmaceuticals are exported by these transporters conferring multidrug resistance onto the cell. This complicates the design of drugs, for instance neural stem cell targeted therapies for the treatment of clinical depression.

7.5 Plasticity

Under special conditions tissue-specific adult stem cells can generate a whole spectrum of cell types of other tissues, even crossing germ layers. This phenomenon is referred to as stem cell transdifferentiation or plasticity. It can be induced by modifying the growth medium when stem cells are cultured *in vitro* or transplanting them to an organ of the body different from the one they were originally isolated from. There is yet no consensus among biologists on the prevalence and physiological and therapeutic relevance of stem cell plasticity (Figure 4).
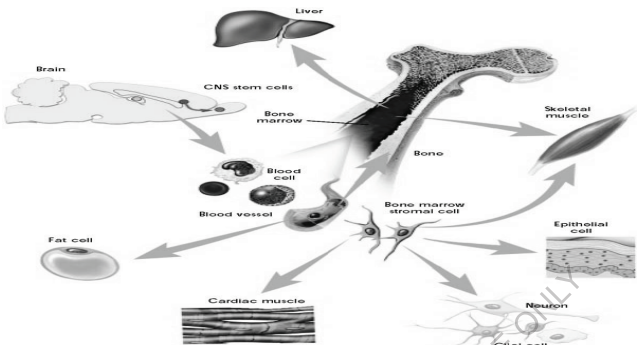
Figure 4. Plasticity of adult stem cells.

7.6 Types of adult stem cells
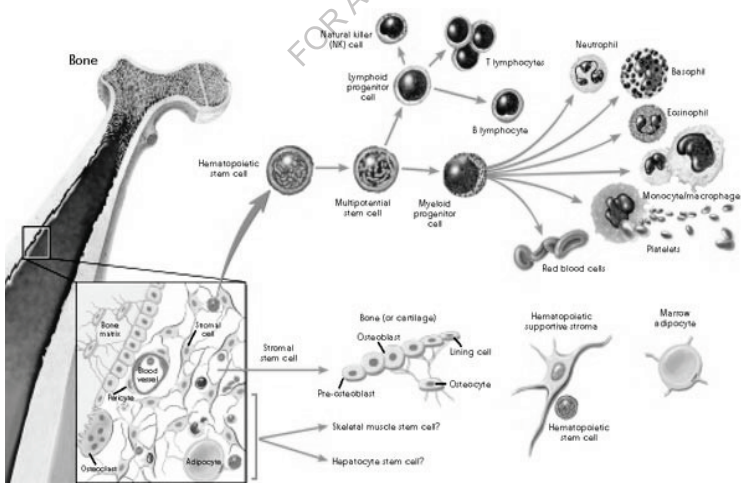
Figure 5. Hematopoietic and stromal stem cell differentiation.

Adult stem cells may also exhibit the ability to form specialized cell types of other tissues, which is known as transdifferentiation or plasticity.

In a living animal, adult stem cells can divide for a long period and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. The following are examples of differentiation pathways of adult stem cells (Figure 5).

Haematopoietic stem cells

Haematopoietic stem cells are found in the bone marrow and give rise to all the blood cell types.

Mammary stem cells

Mammary stem cells provide the source of cells for growth of the mammary gland during puberty and gestation and play an important role in carcinogenesis of the breast. Mammary stem cells have been isolated from human and mouse tissue as well as from cell lines derived from the mammary gland. Single such cells can give rise to both the luminal and myoepithelial cell types of the gland, and have been shown to have the ability to regenerate the entire organ in mice.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are of stromal origin and may differentiate into a variety of tissues. MSCs have been isolated from placenta, adipose tissue, lung, bone marrow and blood, Wharton's jelly from the umbilical cord, and teeth (perivascular niche of dental pulp and periodontal ligament). MSCs are attractive for clinical therapy due to their ability to differentiate, provide trophic support and modulate innate immune response.

Neural stem cells

The existence of stem cells in the adult brain has been postulated following the discovery that the process of neurogenesis, the birth of new neurons, continues into adulthood in rats. The presence of stem cells in the mature primate brain was first reported in 1967. It has since been shown that new neurons are generated in adult mice, songbirds and primates, including humans. Normally, adult neurogenesis is restricted to two areas of the brain - the subventricular zone, which lines the lateral ventricles and the dentate gyrus of the hippocampal formation. Although the generation of new neurons in the hippocampus is well established, the presence of true self-renewing stem cells there has been debated. Under certain circumstances, such as following tissue damage in ischemia, neurogenesis can be induced in other brain regions, including the neocortex.

Neural stem cells are commonly cultured *in vitro* as so called neurospheres - floating heterogeneous aggregates of cells, containing a large proportion of stem cells. They can be propagated for extended periods of time and differentiated into both neuronal and glias cells, and therefore behave as stem cells. However, some recent studies suggest that this behaviour is induced by the culture conditions in progenitor cells, the progeny of stem cell division that normally undergo a strictly limited number of replication cycles *in vivo*. Furthermore, neurosphere-derived cells do not behave as stem cells when transplanted back into the brain.

Neural stem cells share many properties with haematopoietic stem cells (HSCs). Remarkably, when injected into the blood, neurosphere-derived cells differentiate into various cell types of the immune system.

Olfactory adult stem cells

Olfactory adult stem cells have been successfully harvested from the human olfactory mucosa cells, which are found in the lining of the nose and are involved in the sense of smell. If they are given the right chemical environment these cells have the same ability as embryonic stem cells to develop into many different cell types. Olfactory stem cells hold the potential for therapeutic applications and, in contrast to neural stem cells, can be harvested with ease without harm to the patient. This means they can be easily obtained from all individuals, including older patients who might be most in need of stem cell therapies.

Neural crest stem cells

Hair follicles contain two types of stem cells, one of which appears to represent a remnant of the stem cells of the embryonic neural crest. Similar cells have been found in the gastrointestinal tract, Sciatic nerve, cardiac outflow tract and spinal and sympathetic ganglia. These cells can generate neurons, Schwann cells, myofibroblast, chondrocytes and melanocytes.

Testicular cells

Multipotent stem cells with a claimed equivalency to embryonic stem cells have been derived from spermatogonial progenitor cells found in the testicles of laboratory mice by scientists in Germany and the United States, and, a year later, researchers from Germany and the United Kingdom confirmed the same capability using cells from the testicles of humans. The extracted stem cells are known as human adult germline stem cells (GSCs). Multipotent stem cells have also been derived from germ cells found in human testicles.

7.7 Adult stem cell therapies

The therapeutic potential of adult stem cells is the focus of much scientific research, due to their ability to be harvested from the patient. In common with embryonic stem cells, adult stem cells have the ability to differentiate into more than one cell type, but unlike the former they are often restricted to certain types or "lineages". The ability of a differentiated stem cell of one lineage to produce cells of a different lineage is called transdifferentiation. Some types of adult stem cells are more capable of transdifferentiation than others, and for many there is no evidence that such a transformation is possible. Consequently, adult stem therapies require a stem cell source of the specific lineage needed, and harvesting and/or culturing them up to the numbers required is a challenge.

7.7.1 Clinical Applications

Adult stem cell treatments have been used for many years to successfully treat leukemia and related bone/blood cancers utilizing bone marrow transplants. The use of adult stem cells in research and therapy is not considered as controversial as the use of embryonic stem cells, because the production of adult stem cells does not require the destruction of an embryo.

Consequently, the majority of US government funding provided for research in this field is restricted to supporting adult stem cell research.

Early regenerative applications of adult stem cells have focused on intravenous delivery of blood progenitors known as Hematopetic Stem Cells (HSC's). Other early commercial applications have focused on Mesenchymal Stem Cells (MSC's). For both cell lines, direct injection or placement of cells into a site in need of repair may be the preferred method of treatment, as vascular delivery suffers from a "pulmonary first pass effect" where intravenous injected cells are sequestered in the lungs. Clinical case reports in orthopedic applications have been published. Wakitani has published a small case series of nine defects in five knees involving surgical transplantation of mesenchymal stem cells with coverage of the treated chondral defects. Centeno *et al* have reported high field MRI evidence of increased cartilage and meniscus volume in individual human clinical subjects. However, Centeno's stem cell clinic is quite controversial and the results have not been published for more treated patients. Many other stem cell based treatments are operating outside the US, with much controversy being reported regarding these treatments as some feel more regulation is needed as clinics tend to exaggerate claims of success and minimize or omit risks.

7.7.2 First transplanted human organ grown from adult stem cells

In 2008 the first full transplant of a human organ grown from adult stem cells was carried out at the Hospital Clínic of Barcelona on Claudia Castillo, a Colombian female adult whose trachea had collapsed due to tuberculosis. Researchers from the University of Padua and the University of Bristol harvested a section of trachea from a donor and stripped off the cells that could cause an immune reaction, leaving a grey trunk of cartilage. This section of trachea was then "seeded" with stem cells taken from Ms. Castillo's bone marrow and a new section of trachea was grown in the laboratory over four days. The new section of trachea was then transplanted into the left main bronchus of the patient. Because the stem cells were harvested from the patient's own bone marrow Professor Macchiarini did not think it was necessary for her to be given anti-rejection (immunosuppressive) medication and when the procedure was reported four months later in *The Lancet*, the patient's immune system was showing no signs of rejecting the transplant.

7.7.3 Adult stem cells and Cancer

In recent years, acceptance of the concept of adult stem cells has increased. There is now a theory that stem cells reside in many adult tissues and that these unique reservoirs of cells are not only responsible for the normal reparative and regenerative processes, but are also considered to be a prime target for genetic and epigenetic changes, culminating in many abnormal conditions including cancer.

7.8 Stem cell treatments

Medical researchers believe that stem cell therapy has the potential to dramatically change the treatment of human disease. A number of adult stem cell therapies exist, particularly bone marrow transplants that are used to treat leukemia. In the future, medical researchers anticipate being able to use technologies derived from stem cell research to treat a wider variety of diseases including cancer, Parkinson's disease, spinal cord injuries, multiple sclerosis and muscle damage, amongst a number of other impairments and conditions.

However, there still exists a great deal of social and scientific uncertainty surrounding stem cell research, which could possibly be overcome through public debate and future research, and further education of the public.

Stem cells, however, are already used extensively in research, and some scientists do not see cell therapy as the first goal of the research, but see the investigation of stem cells as a goal worthy in itself.

Heart muscle repair
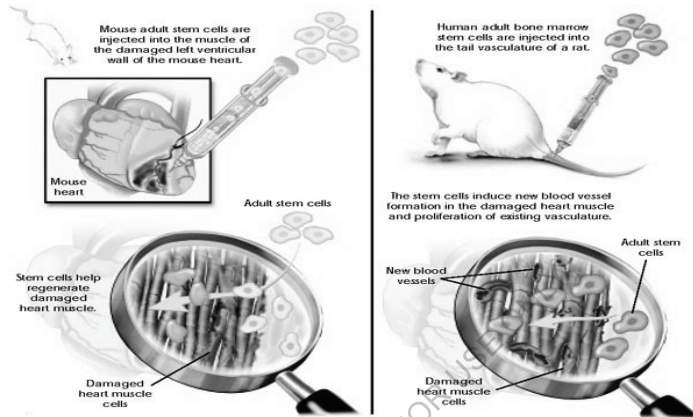
Figure 6. Heart muscle repair with adult stem cells.

For example, it may become possible to generate healthy heart muscle cells in the laboratory and then transplant those cells into patients with chronic heart disease. Preliminary research in mice and other animals indicates that bone marrow stem cells, transplanted into a damaged heart, can generate heart muscle cells and successfully repopulate the heart tissue. Other recent studies in cell culture systems indicate that it may be possible to direct the differentiation of embryonic stem cells or adult bone marrow cells into heart muscle cells (Figure 6).

Organ and tissue regeneration

Tissue regeneration is probably the most important possible application of stem cell research. Currently, organs must be donated and transplanted, but the demand for organs far exceeds supply. Stem cells could potentially be used to grow a particular type of tissue or organ if directed to differentiate in a certain way. Stem cells that lie just beneath the skin, for example, have been used to engineer new skin tissue that can be grafted on to burn victims.

Brain disease treatment

Additionally, replacement cells and tissues may be used to treat brain disease such as Parkinson's and Alzheimer's by replenishing damaged tissue, bringing back the specialized

brain cells that keep unneeded muscles from moving. Embryonic stem cells have recently been directed to differentiate into these types of cells, and so treatments are promising.

Cell deficiency therapy

Healthy heart cells developed in a laboratory may one day be transplanted into patients with heart disease, repopulating the heart with healthy tissue. Similarly, people with type I diabetes may receive pancreatic cells to replace the insulin-producing cells that have been lost or destroyed by the patient's own immune system. The only current therapy is a pancreatic transplant, and it is unlikely to occur due to a small supply of pancreases available for transplant.

Blood disease treatments

Adult hematopoietic stem cells found in blood and bone marrow have been used for years to treat diseases such as leukemia, sickle cell anemia, and other immunodeficiencies. These cells are capable of producing all blood cell types, such as red blood cells that carry oxygen to white blood cells that fight disease. Difficulties arise in the extraction of these cells through the use of invasive bone marrow transplants. However hematopoietic stem cells have also been found in the umbilical cord and placenta. This has led some scientists to call for an umbilical cord blood bank to make these powerful cells more easily obtainable and to decrease the chances of a body's rejecting therapy.

8 Research with stem cells

Scientists and researchers are interested in stem cells for several reasons. Although stem cells do not serve any one function, many have the capacity to serve any function after they are instructed to specialize. Every cell in the body, for example, is derived from first few stem cells formed in the early stages of embryological development. Therefore, stem cells extracted from embryos can be induced to become any desired cell type. This property makes stem cells powerful enough to regenerate damaged tissue under the right conditions.

General scientific discovery

Stem cell research is also useful for learning about human development. Undifferentiated stem cells eventually differentiate partly because a particular gene is turned on or off. Stem cell researchers may help to clarify the role that genes play in determining what genetic traits or mutations we receive. Cancer and other birth defects are also affected by abnormal cell division and differentiation. New therapies for diseases may be developed if we better understand how these agents attack the human body.

Another reason why stem cell research is being pursued is to develop new drugs. Scientists could measure a drug's effect on healthy, normal tissue by testing the drug on tissue grown from stem cells rather than testing the drug on human volunteers.

When does life begin?

The core of this debate - similar to debates about abortion, for example - centers on the question, "When does life begin?" Many assert that life begins at conception, when the egg is fertilized. It is often argued that the embryo deserves the same status as any other full grown human. Therefore, destroying it (removing the blastocyst to extract stem cells) is akin

to murder. Others, in contrast, have identified different points in gestational development that mark the beginning of life - after the development of certain organs or after a certain time period.

Chimeras

People also take issue with the creation of chimeras. A chimera is an organism that has both human and animal cells and tissues. Often in stem cell research, human cells are inserted into animals (like mice or rats) and allowed to develop. This creates the opportunity for researchers to see what happens when stem cells are implanted. Many people, however, object to the creation of an organism that is "part human".

Legal issues

The stem cell debate has risen to the highest level of courts in several countries. Production of embryonic stem cell lines is illegal in Austria, Denmark, France, Germany, and Ireland, but permitted in Finland, Greece, the Netherlands, Sweden, and the UK. In the United States, it is not illegal to work with or create embryonic stem cell lines. However, the debate in the US is about funding, and it is in fact illegal for federal funds to be used to research stem cell lines that were created after August 2001.

9 Stem cell characterization by immunocytochemistry

Following the isolation of cell populations, using differential adhesion or magnetic beads, the cell phenotypes can be characterized immunocytochemically. This can be done in one of several ways, including FACS analysis if available, or the cells can be fixed onto microscope slides, by air drying or use of a cytospin, stained, and examined by fluorescence microscopy. Alternatively, in the absence of a fluorescence microscope, staining can be carried out using a staining method whereby the location of the antibody is visualized by a colour reaction, with a substrate such as 3,3'-diaminobenzidine. One reliable way to use the latter method is with a kit such as the VECTASTAIN Elite ABC kit, from Vector Laboratories. There follows a description of proteins that are useful as markers to distinguish subpopulations of some epithelial cell types, along with methods for the preparation of samples of cells for staining.

9.1 Antibody markers of differentiated cell phenotypes

Cells of all tissues express a wide repertoire of cell surface and cytoplasmic proteins and many of these are cell type-specific, while others show changes of expression during the differentiation of the cells in an epithelium, or *in vitro*.

The cell surface proteins include the integrins, together with a wide number of other members of the CD (cluster of differentiation) antigens such as CD10, CD44, CD31, CD57. One of the major groups of cytoplasmic proteins expressed by epithelial cells is the cytokeratin family and this has proved a useful set of markers of epithelial tissue type.

9.1.1 Keratin markers of epithelial cell type

Cytokeratins form intermediate filaments and are classified as members of either the basic or acidic subfamilies, with one basic cytokeratin always expressed together with an acidic partner. For example keratin 14 is always found co-expressed with cytokeratin 5. In various basal cell layers K5 is also found pairing K15,17, and 19, while K8 pairs with K7, 18,

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and 19. Other pairs include Kl with 10, 4 with 13, and 6 with 16. K19 is unusual in that it can pair with either K5 or K8, depending on its location. The cytokeratins are all relatively abundant and stable, which makes them suitable for immunological detection and, although the members of the keratin family share a high degree of homology, there are sufficient differences for specific antibodies to have been raised against most of them. Some antibodies also recognize a more widely expressed epitope and will see several different cytokeratins. These antibodies include LP34, which will positively stain most cells of epithelial origin and can be considered a pan-cytokeratin antibody.

These are now available from a variety of commercial sources. Two cytokeratins have been shown to stain distinct subpopulations of basal keratinocytes and may prove to be markers for epidermal stem cells. Cytokeratin 19 was shown to stain regions of the hair follicle, individual integrin-bright interfollicular cells, and some cells in culture. As the hair follicle is reported to contain stem cells this may be useful. More recently Lyle *et al* have shown that C8/144B, an antibody originally raised against CD8, a T cell marker, recognizes cytokeratin 15 in a restricted population of hair follicle cells with stem cell characteristics such as label retention and high B1 integrin expression. The lack of cell type specificity of this antibody makes it potentially problematic for use in other tissues and other cytokeratin 15-specific antibodies have shown a wider distribution of staining in the skin.

9.1.2 Cell surface markers of epithelial cell type

There are many different proteins found on the cell surface, ranging from highly abundant adhesion molecules to growth factor receptors and proteins of as yet undetermined function. Cell surface markers are useful tools for the cell biologist because they can be detected by antibodies without prior fixation or permeabilization of the cells. This allows the use of flow cytometry or immunomagnetic beads for cell separation to isolate viable cell populations from tissue. The choice of a suitable cell surface marker for the characterization of experimentally isolated cells depends on several criteria. Some markers will detect all cells of one type, while others may recognize only a differentiated or undifferentiated subset. As the cells will normally have been isolated by trypsinization, the target marker epitope must not be sensitive to trypsin cleavage, a problem with particular antibodies recognizing some transmembrane proteins, such as CD44 and syndecan. Most markers of this type are differentially expressed between cell layers in epithelial tissues such as the skin, breast, and prostate, while some of them are more specific for tissue type, such as MUC-1 in the breast luminal epithelial cells.

9.2 Staining cell suspensions using cytospin preparations

The following protocol is a rapid method to fix cells onto a glass microscope slide for immunostaining. The second part details a method to fix cells onto coverslips for staining without the use of a cytospin. While the second method takes a longer time it is as effective as the first.

10 Stem cell controversy

There exists a widespread controversy over human embryonic stem cell research that emanates from the techniques used in the creation and usage of stem cells. Human embryonic stem cell research is controversial because, with the present state of technology, starting a stem cell line requires the destruction of a human embryo and/or therapeutic cloning.

However, recently, it has been shown in principle that adult stem cell lines can be manipulated to generate embryonic-like stem cell lines using a single-cell biopsy similar to that used in preimplantation genetic diagnosis that may allow stem cell creation without embryonic destruction. It is not the entire field of stem cell research, but the specific field of human embryonic stem cell research that is at the centre of an ethical debate.

Opponents of the research argue that embryonic stem cell technologies are a slippery slope to reproductive cloning and can fundamentally devalue human life. Those in the pro-life movement argue that a human embryo is a human life that is entitled to protection.

Contrarily, supporters of embryonic stem cell research argue that such research should be pursued because the resultant treatments could have significant medical potential. It is also noted that excess embryos created for *in vitro* fertilization could be donated with consent and used for the research.

The ensuing debate has prompted authorities around the world to seek regulatory frameworks and highlighted the fact that stem cell research represents a social and ethical challenge.

The debates surrounding stem cell research primarily are driven by methods concerning embryonic stem cell research. It was only in 1998 that researchers from the University of Wisconsin-Madison extracted the first human embryonic stem cells that were able to be kept alive in the laboratory. The main critique of this research is that it required the destruction of a human blastocyst. That is, a fertilized egg was not given the chance to develop into a fully-developed human.

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Chapter 12
Applications and Economic Implications of Animal Biotechnology

1. Introduction

The developing world is grossly unprepared for the new technological and economic opportunities, challenges and risks that lie on the horizon. Although it is hoped that biotechnology will improve the life of every person in the world and allow more sustainable living, crucial decisions may be dictated by commercial considerations and the socioeconomic goals that society considers to be the most important. Globally, livestock production is growing faster than any other sector, and by 2020 livestock is predicted to become the most important agricultural sector in terms of added value. The use of biotechnology will lead to a distinct shift in the economic returns from livestock. Livestock production currently accounts for about 43% of the gross value of agricultural production. In developed countries livestock accounts for more than half of agricultural production, while in developing countries the share is about one-third. This latter share, however, is rising quickly because of rapid increases in livestock production resulting from population growth, urbanisation, changes in lifestyles and dietary habits and increasing disposable incomes.

The livestock economy in developing countries Livestock is becoming increasingly important in the growth of agriculture in developing economies. The contributions made by livestock to both agriculture and gross domestic product (GDP) have risen, at a time when the contribution of agriculture to GDP has fallen. The demand for livestock products is a function of income, and sustained growth in per capita income, rising urban populations and changes in diet and lifestyle are fuelling growth in livestock production. Livestock production contributes to socioeconomic development in many ways, by augmenting income and employment and reducing the incidence of rural poverty. Though the role of livestock in ensuring nutritional security is recognised in mixed crop-livestock systems, the importance of livestock goes beyond direct food production. Livestock supply draught power and organic manure to the crop sector, and hides, skins, bones, blood and fibre are used in many industries. Thus, livestock are an important source of income and employment, helping to alleviate poverty and smooth the income distribution among small landholders and the landless, which constitute the bulk of the rural population and the majority of livestock owners. In addition, livestock can easily be converted into cash and thus act as a cushion against crop failure, particularly in less favoured environments. By enabling crop residues and by-products to be used as fodder, livestock production contributes positively to the environment. Animal owners in the developing world are predominantly resource-poor small-scale operators with little or no land and few animals, who must operate within the constraints of the local climate and who have limited purchasing power and little access to resources or opportunity to determine resource allocation for animal production. The situation of the poorest livestock owners is fast deteriorating owing to the fragmentation of limited holdings, exhaustion of land resources and increasing human and animal population pressure. Low livestock productivity in many developing countries is considered to reflect, among other things, the inadequate supply of animal husbandry and veterinary services. Veterinary services have traditionally been provided by the State, but financial constraints have limited the availability and effectiveness of public services. The implications of technology A major benefit of agricultural research and technology is that the purchasing power of the poor

increases, because both average incomes and access to staple food products are improved. Studies by economists have provided empirical support for the proposition that growth in the livestock sector affects the whole economy. Rapid growth of livestock production has stimulated demand for and increased the value of land, labour and non-agricultural goods and services, thereby leading to overall economic growth. The poor spend a relatively high proportion of any additional income on food, so increases in livestock production achieved through the use of biotechnology can have major nutritional implications, particularly if the technology is aimed at the poorest producers. However, studies have revealed that the commercialisation of agriculture has reduced the nutritional security of the poor. Once production of milk, meat or eggs has been enhanced through the use of technology, it is hoped that it will also make a significant difference in other areas such as nutrition, prevention of diseases, healthcare and other management practices. It is in these areas that biotechnology shows promise and is currently being used. Green Revolution technologies (i.e., those technologies designed to improve the efficiency of agricultural processes and increase crop productivity by relying on the extensive use of chemical fertilizers/pesticides and heavy machinery) are intended to be used in package form (e.g., new plant varieties supplied with recommendations on fertilizer, pesticide and herbicide rates and water control measures); however, among livestock producers many components of these technologies have been taken up in a piecemeal, often stepwise, manner. The sequence of adoption is determined by availability and by the potential cost savings. The sequential adoptions of crop management technologies for rice and wheat have been assessed in detail, but few similar studies have focussed on livestock production in developing countries. Evidence from the People's Republic of China, Mexico, South Africa and India suggests that small farmers have had no more difficulty than larger farmers in adopting the new technologies. The question, therefore, is not whether biotechnology can benefit small-scale resource-poor farmers, but rather how biotechnology can address the agricultural problems faced by farmers in developing countries. Biotechnology is a promising new tool in the development of applied agricultural technologies. The challenge is to focus this potential on the problems experienced by developing countries. The introduction of multi-enterprise systems or, more broadly, agricultural diversification is seen as the way forward for agriculture in the developing world; such systems could lift small-scale and marginal farmers out of poverty. For example, rotating rice and wheat cropping with dairy farming yields higher profits. Introducing multi-enterprise systems involving livestock enhances the purchasing power of farmers and helps them to obtain nutritional security. It also generates rural (both farming and non-farming) employment, thus preventing excessive migration to urban areas, which is a common problem in developing economies. Multi-enterprise systems also support the natural environment and contribute to capital formation, thus leading to higher overall growth in the agricultural economy. The technology, infrastructure and institutions now exist to make the application of biotechnology in the context of a multi-enterprise system involving livestock production economically viable.

2.Global advantage from livestock of developing Countries

The multiplicity of genes, species, populations and agroecosystems in the developing countries of South and South East Asia, the People's Republic of China, Africa and Latin America is viewed as a valuable resource for the genetic improvement of livestock on a global scale. The livestock in these countries, which are an integral part of a fragile ecosystem, are a rich source of animal biodiversity. Buffaloes, sheep, goats, camels and zebu cattle have adapted to their regional environments over thousands of years and have provided an important source of sustenance for the population of the region. Livestock production in

the developing world has a number of advantages over production in more developed countries, for example:

- the unique and valuable production traits of buffaloes, cattle, sheep, goats and camels
- the low-input production system
- the low unit cost of production
- the lean meat produced from sheep, goats and buffaloes
- the considerable biodiversity
- animal breeds that are resistant to stress and to particular diseases
- the ability of the animals to survive on high-roughage feeds
- the potential for biopharmaceutical developments to lead to significant benefits
- the potential for expanding the microbial food, feed and leather industries
- the integrated production system tailored to the local ecology
- the potential for integrating knowledge and industry.

Several genes and desirable traits have been identified in the livestock of developing countries in Asia and Africa, and some of the livestock species and breeds from these countries have become major contributors to the economy of South America. Examples of breeds from the developing world that are particularly important on a global level are:

- Buffaloes that produce milk with a high fat content or with the protein quality required to produce mozzarella cheese
- Goats from cold dry regions that produce pashmina and toos (the finest wool in the world)
- Black Bengal goats that carry a gene for high prolificacy
- Garole sheep that carry genes for twinning
- Andaman goats that is highly tolerant of salt
- The yak and mithun that are adapted to high altitude
- The camels, sheep and goats that are adapted to a tropical arid environment and can tolerate feed with a high lignin content
- The many species those are resistant to stress or to particular diseases.

3. Economic impact of technologies

The genetic resources possessed by animals in developing countries often affect economic development. The economic impacts of biotechnological innovations, but the research and policy options need separate consideration. Animal biotechnology is the result of a multistage process, involving research, development, testing and registration, production and marketing. The goal is to develop a technology, process or product that has clear commercial potential and can be commercialised after due testing and regulatory approval. Developing countries find it difficult to develop biotechnology because the facilities or resources needed to complete all of the stages in the process are often lacking. However, several technologies from developed countries have been successfully adopted by developing countries. The impact of technology can be analysed by estimating the growth of total factor productivity (TFP) in livestock production. Not many TFP studies on livestock have been reported. However, separate TFP estimates for the aggregate crop and livestock sectors have been made. TFP analysis has shown a shift towards larger, more commercial and more intensive production systems and has further revealed that, as specialisation has developed over the past decade, the importance of backyard livestock production has declined and the

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importance of specialised household and commercial enterprise has increased. Studies from India have shown that technological input is responsible for about 45% of total output growth and that the TFP growth may be as much as 1.8%.

Technologies that have a specific impact in developing countries There are a large number of technologies that have been developed for or adapted to the livestock of both developed and developing countries. However, the major technologies that are used effectively in livestock production in the developing world include conserving animal genetic resources, augmenting reproduction, embryo transfer (ET) and related technologies, diagnosing disease and controlling and improving nutrient availability.

4. Transgenics

Although gene-based technologies have the potential to improve the efficiency of livestock production, thereby ensuring better returns for the farmers, the economic impact of transgenics in the livestock sector will be much less than in the crop sector. However, the global adoption of genetically modified (GM) crops, which were grown on 67.7 million hectares in 2003 compared with 2.8 million hectares in 1996, has had a substantial impact on livestock feed. It is estimated that the United States of America (USA), Argentina, Canada, Brazil and the People's Republic of China have 63%, 21%, 6%, 4% and 4%, respectively, of the global transgenic acreage and that the most frequently grown crops are GM soybean (61%), maize (23%), cotton (11%) and canola (5%). Although few developing countries have released GM crop varieties, a preliminary analysis reveals that more than 20 developing countries are conducting research into the applications of GM crops. Although transgenic animals (especially mice) are used routinely in research (particularly in the medical field), no GM animals have yet been released on farms. A wide range of traits of potential interest to livestock producers have, however, been the subject of research; for example, the gene responsible for the production of growth hormone (which could be manipulated to increase growth rates), the phytase gene (which could reduce phosphorous emissions from pigs) and keratin genes (which could improve the wool of sheep). The genetic modification of livestock has proceeded much more slowly than the genetic modification of crops for a variety of reasons, including the high costs, the inefficiency of the gene transfer techniques and the low reproductive rates of animals. Recombinant deoxyribonucleic acid (DNA) approaches have been used to promote the expression of desirable genes, to hinder the expression of undesirable genes, to alter specific genes and to inactivate genes so as to block specific pathways. It is estimated that at least 30 enzymes produced by GM bacteria, yeasts and moulds are currently commercially available worldwide; many of these enzymes are used in the food industry. Genetic engineering has been used to introduce foreign genes into the animal genome or, alternatively, to knock out selected genes. Genes controlling growth were introduced into pigs to increase growth and improve carcass quality. Currently, research is underway to engineer resistance to diseases that affect the animals or that pose an indirect risk to human health, such as Marek's disease and salmonellosis in poultry, scrapie in sheep and mastitis in cattle. Other studies have tried to increase the casein content of milk or to engineer animals that produce pharmaceutical or industrial chemicals in their milk or semen. No agricultural applications have yet proved commercially successful. Nuclear transfer (NT) technology now provides an alternative route for cell-based transgenesis in domestic species, offering new opportunities for genetic modification. Livestock that produce human therapeutic proteins in their milk, that have organs suitable for xenotransplantation and that are resistant to diseases such as spongiform encephalopathies have been produced by NT from engineered cultured somatic cells.

5. Characterising genetic variability

There is considerable genetic diversity in the livestock of developing countries, much of which controls traits that influence adaptability to harsh environments, productivity and susceptibility to disease and parasitism. However, little if any data on these genetic resources are available.

Economic analysis can play an important role in ensuring that conservation efforts are appropriately focused. The primary challenge facing conservationists is to identify sound reasons why society should preserve animals that livestock keepers have abandoned. Jabbar and Diedhiou show that the breeding practices and breed preferences of livestock keepers can successfully be determined by using research techniques such as the revealed preference hedonic approach. On the one hand, conservation cannot be achieved through a conventional breeding programme because the animals carrying the most advantageous traits cannot be easily identified; Conversely, conservation cannot be achieved through biotechnology because the necessary technologies are either unavailable or uneconomic. In livestock populations with a high degree of genetic variation, molecular markers are being increasingly used to study the distribution and patterns of genetic diversity. Global surveys indicate that 40% of domestic livestock breeds are at risk of extinction. Most of these breeds are found only in developing countries, and often little is known about them or their potential. Rapid progress is being made in the preparation of dense microsatellite linkage maps to assist in the search for genetic traits of economic importance. These linkage maps can be used to develop strategies for marker assisted selection and marker assisted introgression that will meet the goals of breeding programmes in developing countries. Molecular markers have been widely used in the identification of genotypes and the 'genetic fingerprinting' of organisms. Genotype verification is used intensively to determine the parentage of domestic animals and to trace livestock products in the food chain back to the farm and animal of origin.

6. Reproductive technologies

The main objectives of using reproductive biotechnologies in livestock are to increase production, reproductive efficiency and rates of genetic improvement. Over the years, many options have become available for managing the reproduction of the major large and small ruminants. Artificial insemination (AI) and preservation of semen are the main technologies that are used extensively. Assessing the fertilisation capacity of sperms, sexing sperms, synchronisation and fixed-time insemination, superovulation, embryo transfer (ET) and *in vitro* embryo production (IVEP) are additional techniques that can improve reproductive efficiency and pregnancy rates. Reproductive technologies can also be used to control reproductive diseases if procedures and protocols are accurately followed.

6.1 Artificial insemination

The conception rate in field AI programmes in developing countries is very low, and therefore the desired effect in terms of animal improvement has not been achieved. Most semen banks still evaluate semen on the basis of sperm motility, even though significant advances have been made in techniques for semen evaluation. Although detailed guidelines are available regarding the processing, storage and thawing of cattle semen and buffalo semen, the processing and handling procedures in laboratories processing semen are often inadequate. Only when farmers have access to considerably better technical and

organisational facilities will AI become more effective. At present, the efficiency of the technology is limited by organisational and logistical constraints and by the failure to provide appropriate training for farmers. Several modifications of the technique have been suggested to increase the conception rate. Synchronisation with different compounds, and the use of gonadotropin-releasing hormone (GnRH) followed seven days later by prostaglandin F2α (PGF2α) can synchronise oestrus and improves the conception rate. In this protocol giving injections of GnRH on day 0, PGF2α on day 7 and GnRH on day 9 is called the 'Ovsynch' programme and synchronises ovulation, permitting timed insemination. The ability to control ovarian follicular and corpus luteum development has allowed insemination in cattle to be timed without having to detect oestrus, and this has increased the net revenue per cow.

6.2 Embryo transfer

One of the major reproductive technologies that can facilitate genetic improvement in cattle is ET. Unfortunately, commercial ET programmes are limited by the high variability in the ovarian follicular response to gonadotropin stimulation. Multiple ovulation and embryo transfer (MOET) takes AI one step further, in terms of both the possible genetic gains and the level of technical expertise and organisation required. In 2001, 450,000 embryos were transferred globally, mainly in dairy cattle, with 62% being transferred in North America and Europe, 16% in South America and 11% in Asia. The main potential advantage of MOET for developing countries is that the elite females of local breeds can be identified, and bulls can be produced from them for use in a field programme of breed improvement. Zebu cattle and buffaloes in developing countries exhibit less consistent follicular dynamics after superovulation than *Bos taurus* in the developed world. However, over the last 10 to 15 years, the number of transferable embryos produced by zebu donors has increased from 2.4 to 5.8 embryos per flush in the late 1980s to 5.6 to 9.9 embryos per flush in 2000. The use of ET has been less successful than envisaged for several reasons. The low reproductive efficiency, poor superovulatory responses, very low primordial follicle population and high incidence of atresia all contribute to low embryo production. In buffaloes, embryo recovery was initially less than one, but has subsequently improved to 2.6 with 1.4 transferable embryos per flush. After transferring buffalo embryos to recipients, the conception rate is only 16%. The poor success rates have limited the use of ET in buffaloes, which are the main dairy animals in developing countries in Asia, South-East Asia and the Mediterranean region. *In vitro* production of embryos since the birth of the first buffalo calf from an *in vitro* fertilised oocyte, a number of publications have described the effects of different protocols and media on oocyte and embryo development. Two extensive reviews have been published recently. However, the practical use of IVEP is limited by high production costs and the low overall efficiency under field conditions. High rates of maturation (70% to 90%), fertilisation (60% to 70%) and cleavage (40% to 50%), and moderate to low rates of blastocyst formation (15% to 30%) and calf production (10.5%) have been reported. The efficiency of blastocyst production in buffaloes is much poorer than the 30% to 60% reported for cattle. Although viable buffalo blastocysts have been produced from ovaries obtained from abattoirs, the yield of transferable embryos remains low (15% to 39%). Embryos produced *in vitro* have led successfully to pregnancy and calf birth in buffalo, but the success rate is low. Therefore IVEP must be improved before it can be widely used in cattle and buffaloes in developing countries.

7. Improving health through developing vaccines

Most biotechnologies related to health focus on the needs of the developed world, meaning that 90% of health research is devoted to the health problems of 10% of the world's population. Two main approaches are being used to develop vaccines using recombinant DNA technology. The first involves deleting genes that determine the virulence of the pathogen, thus producing attenuated organisms (non-pathogens) that can be used as live vaccines. Currently, this strategy is more effective against viral and bacterial diseases than against parasites. Attenuated live vaccines have been developed against the herpes viruses that cause pseudorabies in pigs and infectious bovine rhinotracheitis in cattle. A number of candidate *Salmonella* vaccines have also been produced. The second approach is to identify protein subunits of pathogens that can stimulate immunity. The International Livestock Research Institute (ILRI) used this approach to develop a vaccine against *Theileria parva*, the parasite that causes East Coast fever in African cattle. A novel strategy for developing vaccines against bloodsucking parasites involves using components of the gut wall of the parasite that are not usually exposed to the immune system of the host. When the parasite feeds, it ingests antibodies induced by the vaccine, which destroy the gut wall and, consequently, kill the parasite. This strategy has been used successfully to develop a vaccine against the one-host tick *Boophilus microplus*. Vaccination is one of the most effective and sustainable methods of controlling disease. Vaccines against parasitic diseases in Africa and viral diseases in Asia have been shown to control disease effectively and increase livestock productivity. A recent approach has been to use vaccines based on DNA. The use of DNA in vaccines is based on the discovery that injecting genes in the form of plasmid DNA can stimulate an immune response to the respective gene products. This immune response is a result of the genes being taken up and expressed by cells in the animal after injection. The live-vector and DNA vaccination systems could be manipulated further to enhance the immunity conferred by the gene products. Experimental studies have demonstrated that these vaccines can potentially induce appropriate and enduring immune responses. This technology is, in principle, one of the simplest and yet most versatile methods of inducing both humeral and cellular immune responses, as well as protecting against a variety of infectious agents. However, although immune responses have been induced in a number of larger species, most of the information on the efficacy of DNA immunisation comes from studies of mice.

An exhaustive review of the information available on the use of DNA vaccines in farm animals, including cattle, pigs and poultry, has identified the areas that need specific attention before this technology can be used routinely. These areas include the delivery, safety and compatibility of plasmids in multivalent vaccines and the potential for using immune stimulants as part of a DNA vaccine. Korean scientists have developed a combined vaccine against pleuropneumonia, pneumonic pasteurellosis and enzootic pneumonia in swine. Molecular biology has been used to produce an improved vaccine against swine fever. In the Philippines, a vaccine has been developed that protects cattle and water buffalo against haemorrhagic septicaemia, which is the leading cause of death in these animals. The new vaccine provides improved protection at a very low cost.

8. Diagnostics and epidemiology

Advanced diagnostic tests that use biotechnology enable the agents causing disease to be identified and the impact of disease control programmes to be monitored more precisely than was previously possible. Molecular epidemiology characterises pathogens (viruses, bacteria, parasites and fungi) by nucleotide sequencing, enabling their origins to be traced. This is particularly important for epidemic diseases, in which pinpointing the source of the infection can significantly improve disease control. For example, the molecular analysis of

rinderpest viruses has been vital in determining the lineages circulating in the world and instrumental in aiding the Global Rinderpest Eradication Programme. Enzyme-linked immunosorbent assays have become the standard means of diagnosing and monitoring many animal and fish diseases worldwide, and the PCR technique is especially useful in diagnosing livestock disease. Many diagnostic techniques currently used in developing countries are cumbersome and unsuitable for low-resource settings. Molecular diagnostic technologies that are either already in use or being tested in low-income regions include polymerase chain reaction (PCR), monoclonal antibodies and recombinant antigens. These technologies can be modified to facilitate their application in the developing world. Simple hand-held devices that rely on the binding specificity of monoclonal antibodies or recombinant antigens to diagnose infection may be easily adapted for use in settings without running water, refrigeration or electricity.

Molecular characterisation of the virus serotypes causing foot and mouth disease has helped in the vaccination and control programmes in Asia. In Japan and Taiwan, DNA testing is being used to diagnose hereditary weaknesses of livestock. One test looks for the presence of the gene responsible for porcine stress syndrome in pigs. Pigs with this gene tend to produce pale poor-quality meat because of their reaction to the stress of transport and slaughter. Pigs with this gene can now be excluded from breeding programmes, so the gene will become less common. In addition, DNA testing is being used in Japan to check for the gene that causes leucocyte adhesion deficiency in Holstein cattle. Cattle with this condition suffer from gum disease, tooth loss and stunted growth. They usually die before they are one year old. By using DNA testing, carriers can be identified and eliminated from breeding herds. Bulls used for breeding can also be tested to make sure that they are not carriers. Another DNA test identifies a gene that leads to anaemia and retarded growth in Japanese Black cattle.

9. Nutrition and feed utilisation

The shortage of feed in most developing countries and the increasing cost of feed ingredients mean that there is a need to improve feed utilisation. Aids to animal nutrition, such as enzymes, probiotics, single-cell proteins and antibiotics in feed, are already widely used in intensive production systems worldwide to improve the nutrient availability of feeds and the productivity of livestock. Gene-based technologies are being increasingly used to improve animal nutrition, either through modifying the feeds to make them more digestible or through modifying the digestive and metabolic systems of the animals to enable them to make better use of the available feeds. Feeds derived from GM plants (a quarter of which are now grown in developing countries), such as grain, silage and hay, have contributed to increases in growth rates and milk yield. Genetically modified crops with improved amino acid profiles can be used to decrease nitrogen excretion in pigs and poultry. Increasing the levels of amino acids in grain means that the essential amino acid requirements of pigs and poultry can be met by diets that are lower in protein. Metabolic modifiers have also been used to increase production efficiency (weight gain or milk yield per feed unit), improve carcass composition (meat-fat ratio), increase milk yield and decrease animal fat. The use of recombinant bovine somatotropin (rBST) in dairy cows increases both milk yield and production efficiency and decreases animal fat. In the USA, the use of rBST typically increases milk yield by 10% to 15%. Although trials conducted in developing countries have reported a similar percentage increase, this increase is not significant because of the low milk yields and the high cost-benefit ratio. However, rBST is being used commercially in 19 countries where the economic returns make its use worthwhile. A porcine somatotropin has

been developed that increases muscle growth and reduces body-fat deposition, resulting in pigs that are leaner and of greater market value. Constraints on applying the technology The application of new molecular biotechnologies and new breeding strategies to the livestock breeds used in smallholder production systems in developing countries is constrained by a number of factors. In the developing world, poverty, malnutrition, disease, poor hygiene and unemployment are widespread, and biotechnologies must be able to be applied in this context. Over the last few decades, the green revolution has brought comparative prosperity to farmers with land, but the majority of farmers, who are landless or marginal farmers and subsist only on livestock, have been neglected and remain poor. The major constraints on applying biotechnologies have been enumerated and include:

a. the absence of an accurate and complete database on livestock and animal owners so that programmes can be implemented
b. the biodiversity present within species and breeds in agro-ecological systems
c. the fact that models of biotechnological intervention differ distinctly between developed and developing economies
d. the fact that many animal species and breeds are unique to the developing world; each has its own distinct developmental, production, disease resistance and nutrient utilisation characteristics
e. the lack of trained scientists, technicians and fieldworkers to develop and apply the technologies, both in the government and in the private sectors
f. the absence of an interface between industry, universities and institutions, which is necessary to translate technologies into products
g. the inability to access technologies from the developed world at an affordable price to make a rightful, positive and sustainable contribution to livestock production and the economic welfare of farmers
h. the high cost of technological inputs such as materials, biologicals and equipment
i. the failure to address issues of biosafety and to conduct risk analyses of new biologicals, gene products, transgenics and modified food items, and, above all
j. the negligible investment in animal biotechnology.

The critical issues affecting livestock productivity have recently been re-examined. Research that aims to enhance productivity and sustainability should focus on improving livestock feeds and nutrition, improving animal health, managing natural resources relating to the livestock sector, assesing the impact of technological interventions, and strengthening the capacity of the national agricultural research systems of developing countries.

Furthermore, the potential production capacity and contribution of livestock to the economy are still not being achieved in developing countries because the transfer, adaptation and adoption of technology is hampered by the lack of a clear policy for livestock development that is conducive to the introduction of new proven technology and by the lack of information flow from and to decision makers.

In developing countries, there is a wealth gap between urban and rural areas, which persists and may even be widening; the rural-urban divide also tends to be reflected in education and health indicators. In addition, women in rural (and urban) areas who are predominantly involved in animal husbandry have higher illiteracy rates than men. A survey of 21 African countries recently highlighted the substantial disparities in primary schooling between urban and rural areas, in favour of urban dwellers. Special attention must be given to the knowledge and information needed to enable rural people to apply biotechnology. There

is a need to identify alternative delivery systems (beyond the State) for animal healthcare and to propose new roles for the state and the private sector in service delivery.

10. Building capacity

Owing to the constraints outlined above, the economic benefits of animal biotechnology cannot be realised without a conscious, sustained, holistic, multi-stakeholder, participatory approach. There is a great need to ensure that capacity is not just created but also is retained and enhanced. Capacity-building activities must be carried out at all levels: the awareness of policy and decision makers must be raised, the necessary legal and regulatory frameworks must be initiated, the technical and regulatory capacities must be enhanced and institutions may need to be overhauled. More importantly, it is necessary to assess and deploy competent operators and institutional capacity continuously so that, as biotechnology advances, the procedures required for its safe use can be constantly evaluated, upgraded and applied. This is a daunting task, but it can be achieved through firm commitment and partnerships.

11. Funding to implement technology

Developing and commercialising improved technologies in most developing countries has been the responsibility of the public sector, and technology has been disseminated freely. This situation will have to continue if superior genetics, diagnostics and vaccines are to be delivered. However, research and most commercial development of biotechnology in the developed world are being driven largely by the private sector. The global trends in funding for research and development and production do not address the concerns, needs and opportunities of the developing world. Developing countries are finding it increasingly expensive to access and use new technologies. There is limited private and public-sector investment in animal health and production, particularly in relation to modern biotechnologies that are 'resource hungry'. Although several discoveries have been made in laboratories in the developing world, in most cases these have not been converted into useful technologies or products. The key potential users-resource-poor often illiterate farmers with a limited knowledge base – do not feel that applying these technologies is worth the effort, cost and risk involved. This is mainly because there is no agency or industry that can scale up and package the technology. Also, in the developed world, there is an economic incentive to market biotechnological services and products; this is lacking in the developing world because of the limited purchasing power of resource-poor stakeholders. Research in biotechnology in recent years has also been motivated by economic considerations, and little research is conducted in the developing world because of the probable lack of returns on the investment. For understandable reasons, current funding policies in developing countries focus on areas that will yield practical benefit in the short term. In determining future policy, policy-makers and funding bodies must not lose sight of the substantial benefits that can be gained in the longer term by investing in strategic research into vaccine development. Adequate multi-institutional (national and international) support through an international donor consortium is needed to develop cost-effective, cheap and easily adaptable biotechnological products. The amount spent by international agencies on animal biotechnology in developing countries is currently very low and constitutes only a small percentage of the total spending on agriculture. The World Bank, the Food and Agriculture Organization, the Consultative Group on International Agricultural Research, the United Nations Development Programme, the United States Agency for International Development, the Swedish International Development Cooperation Agency, the International Development

Research Centre, the Asian Development Bank and other donor and funding agencies have to designate a higher percentage of funds to the livestock sector. It has been convincingly shown that investing in livestock has a dramatic and far-reaching impact on the human development index. This is a strong argument in favour of investing heavily in animal production and health biotechnologies to bring economic prosperity, nutritional security, and rural development and health improvements to poor populations in the developing world.

12. Conclusions

Although animal production is being changed significantly by advances made in thousands of biotechnology laboratories around the world, benefits are reaching the developing world in only a few areas of conservation, animal improvement, healthcare (including diagnosis and control of disease) and the augmentation of feed resources. Adopting biotechnology has resulted in distinct benefits in terms of animal improvement and economic returns to the farmers. Over the past decade, the ILRI has focused on biotechnological applications, especially in Africa, and several developing countries now have multi-institutional programmes to develop and apply biotechnology. The developing world will have to respond to the many genebased technologies now being developed with a sense of commitment, trained manpower, infrastructure and funding.

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Chapter 13
Cloning

1. Nuclear transfer

Dolly was not created in the ordinary way. Typically, a lamb is the product of natural reproduction—two germ cells, a sperm from an adult male and an egg (oocyte) from an adult female, fuse at fertilization. Each of these germ cells (the sperm and the oocyte) contributes half the chromosomes needed to create a new individual. Chromosomes are found in the cell's nucleus and they carry the DNA, which is the genetic blueprint for an individual (Fig.1).
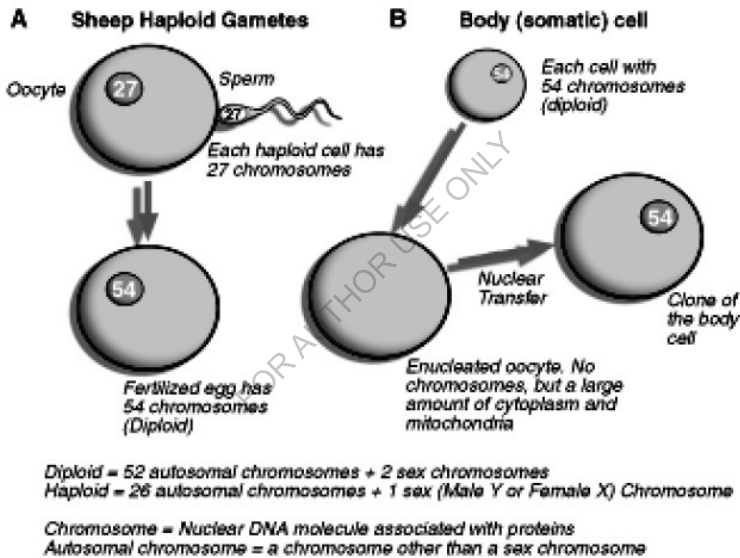
Fig.1 A.Sexual Reproduction and B. Somatic cell nuclear transfer in Sheep

2. Nuclear replacement

Genetic material (nucleus from embryonic, fetal, or adult cell) is removed and placed into an unfertilized egg or embryo, whose nucleus has been removed. In this case the nuclear genes remain the same but the mitochondria DNA would be different. This has the potential to create the clone of an adult organism as well as many clones at once.

Dolly was cloned using the nuclear replacement method. Again the nucleus with chromosome sets is fused with an unfertilized egg whose nucleus has been removed. Motivating factor was that it could help to improve certain qualities in livestock.

Dolly was not the first sheep to be created from nuclear replacement. Two genetically identical sheep, Megan and Morag were born in 1996 using the technique. The difference was that Dolly was derived from an adult sheep, and Megan and Morag were from a sheep embryo

3. Cloning of Dolly

In 1997 Dolly the sheep became the first vertebrate cloned from the cell of an adult animal. Not only was this remarkable scientific breakthrough but it immediately gained interest and concern from around the world on the future of cloning technology as it would affect humans.

Dolly made front pages around the world because of her startling pedigree: Dolly, unlike any other mammal that has ever lived, is an identical copy of another adult and has no father. She is a clone, the creation of a group of veterinary researchers. That work, performed by Ian Wilmut and his colleagues at the Roslin Institute in Edinburgh, Scotland, has provided an important new research tool and has shattered a belief widespread among biologists that cells from adult mammals cannot be persuaded to regenerate a whole animal. Although the Scottish researchers have made clear that they would consider it unethical to adapt their technique to clone humans (Wilmut is a member of a working group on the ethics of genetic engineering), the demonstration has raised the uncomfortable prospect that others might not be so scrupulous.

The process that produced Dolly differs from ordinary reproduction in two major ways. First, body (or somatic) cells from an adult ewe's udder (this is the donor) were placed in a culture dish and allowed to grow. The nutrients were then removed from the culture, which stopped the cells' growth. One of these non-growing cells was then fused (by electric jolts) with another ewe's oocyte from which the nucleus had been previously removed (i.e., enucleated, so it had no chromosomes). This procedure is known as 'somatic cell nuclear transfer'. Within a day the fused cells began to divide in the culture dish. After several divisions, the early embryo was transferred to the uterus of a surrogate mother and allowed to develop.

Second, unlike the sperm and the egg, each of which contributes half the number of chromosomes at fertilization, each body cell contains twice the number of chromosomes in each germ cell. So fusion of a sperm and an egg forms an individual whose full genetic composition is unique to that individual. Conversely, the embryo cloned from somatic cell nuclear transfer begins development with the diploid (double) number of chromosomes, all derived from one somatic cell (adult udder) of a single individual. This embryo has the same nuclear genetic composition as the donor of the somatic cell. In the end, three sheep contributed to the production of a single lamb clone: a Finn Dorset sheep donated her udder cells for culture; a Scottish Blackface sheep donated the enucleated oocyte (with its nucleus removed, thus losing its own genetic identity in the process); and a Scottish Blackface sheep became the surrogate mother, carrying the embryo to birth (Fig.2).
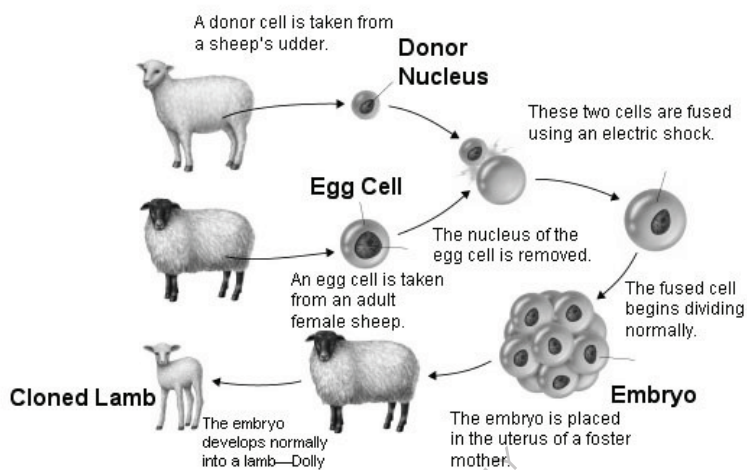
Fig.2 Cloning of Dolly
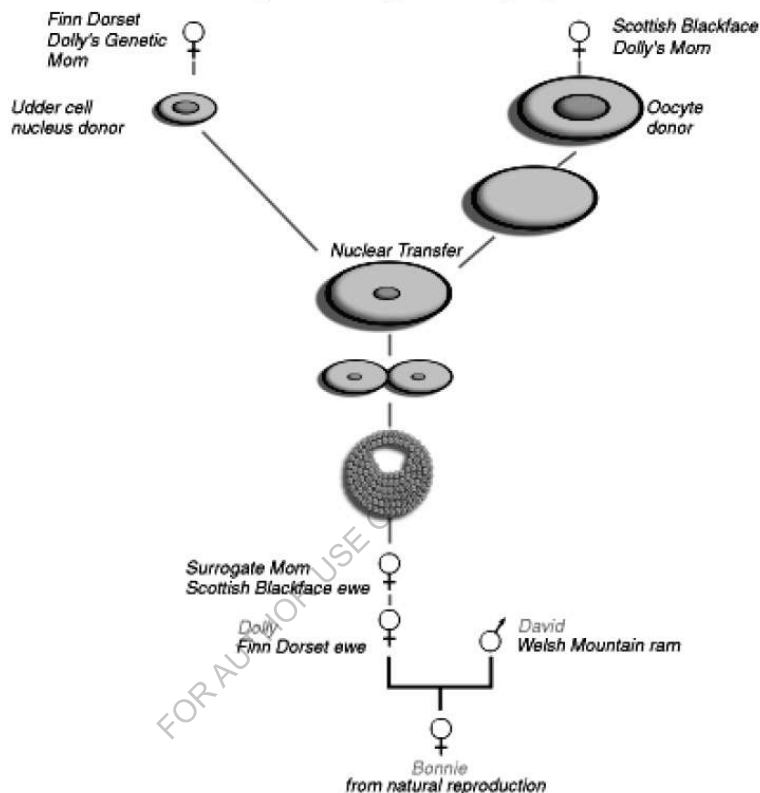
Fig.3 Dolly's Geneology and Offspring

The clone (Dolly) was easily identified because she had the physical traits of the Finn Dorset sheep that donated the udder cells and differed from the traits of the Scottish Blackface sheep used as the surrogate mother and the oocyte donor. And now Dolly herself is a mother-the old-fashioned way-by mating with David (Fig.3), a Welsh mountain ram, and giving birth to Bonnie (Fig.4). In fact, Bonnie now has other siblings.

Fig.4 Dolly with her first son, Bonnie

4. End of Dolly

On February 14, 2003 Dolly was put to sleep. She was 6 years old, and had been diagnosed with progressive lung disease.

4.1 Was it an early death, was it because she was a clone?

Sheep live 11-12 years and Dolly came from a 6 years old ewe. The telomerases were shorter (as the aging prolongs, telomerases become shorter in length).

Lung disease is common in older sheep. She had arthritis on her hind legs.

5. Dolly's probability

- Cells taken from a six-year-old Finnish Dorset ewe and cultured in a lab.
- 277 cells then fused with 277 unfertilized eggs (each with the nucleus removed)
- 29 viable reconstructed eggs survived and were implanted in surrogate Blackface ewes.
- 1 gave birth to Dolly
- 0.361% chance at onset, 3.4482% once implanted. In nature between 33-50% of fertilized eggs develop.

6. Can Dolly help Jimmy, Christine, and Margo?

Imagine herds of female sheep, cattle, and goats producing large quantities of human proteins in their milk, an ideal place for those proteins to be harvested and used to treat patients like Jimmy, the hemophiliac, whose blood cannot clot. We can realize this dream today-one step at a time, because the process that produced Dolly also can be used to produce the transgenic (one species carrying another species' genes) clones.

Scottish scientists first removed cells from a fetal lamb and grew them in a culture dish. Multiple copies of fragments of DNA (deoxyribonucleic acid, which holds genetic information) containing the human gene for blood clotting factor IX were added to the dish and coaxed into the cells. Some cells incorporated the human DNA into their chromosomes, thus becom becoming 'transgenic cells', or cells containing a transferred gene. These transgenic cells were then separated from those without human DNA and used to create Polly, the transgenic sheep that today produces the human clotting factor IX in her milk. Purposely, scientists genetically designed the transgenic sheep clones so that the human gene would function only in the mammary gland. It will soon be possible for the human clotting factor IX protein to be routinely harvested and purified from the sheep milk. Obviously, researchers still need to conduct controlled clinical studies before this protein is available for hemophiliacs like Jimmy, but they have already made an astonishing breakthrough (Fig.5).
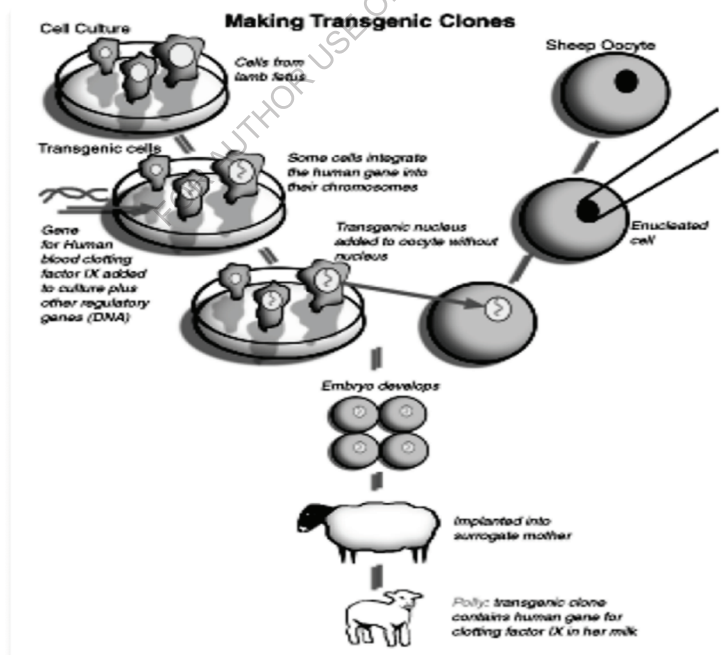
Fig. 5 Making Transgenic Clones

7. Summary-Dolly Cloning

Ian Wilmut and his colleagues at Scotland's Roslin Institute stunned the scientific community by announcing the first cloning of a mammal: Dolly the lamb. Concern about the potential application of similar cloning technology to humans quickly spread through many diverse communities. In response, a multidisciplinary panel consisting of members of the scientific, medical, legal, and ethics communities convened at Stanford University on March 5, 1997, for a public discussion.

Dolly was created by the fusion of an adult ewe's mammary cell ("donor cell") with an enucleated, unfertilized ewe egg ("oocyte").1 The donor cell supplies the diploid genomic DNA, and the oocyte supplies the maternal factors needed for proper embryonic development. Before fusion, the donor cell is cultured with very few nutrients, resulting in arrest of the donor cell in an inactive, or quiescent, state. The DNA of the quiescent donor cell is thus "reprogrammed," because gene expression involved in determining cellular identity is turned off.

Fusion of the donor cell and oocyte is induced using a series of electric pulses. These pulses also simulate the activation signals normally associated with fertilization, which causes the newly fused cell to initiate embryonic development. The developing embryo is cultured; if it survives and continues to develop after six days of culture, it is transferred to a recipient ewe. The embryo is then carried to term. Out of twenty-nine implanted embryos produced by this method, only one resulted in a live birth.

Dolly's birth raises interesting scientific questions. For instance, it remains unclear which types of adult cells have the ability to function as donor cells. Adult mammalian cells can be at varying stages of identity determination ("differentiation"). Cells that are in the early stages of differentiation may be more susceptible to the cloning procedure's reprogramming step. As the Roslin scientists do not know the exact identity of the donor cell that contributed the genomic DNA to Dolly, the extent of de-differentiation required to sufficiently reprogram a donor cell is unknown. Thus, the types of adult donor cells useful in the cloning procedure may be limited.

The age of the donor cell may also limit the ability of the donor cell's genomic DNA to direct normal development. During the aging process, cells naturally acquire genetic mutations; these mutations are often "silent," and have little overt physiological effect in an adult. Such mutations, however, could be devastating in an early embryo. The accumulated mutations of an older donor cell could arrest embryonic development, or result in severe developmental abnormalities in the clone. A donor cell from an eighty-year-old human may therefore be dangerous to use in cloning.

Although Dolly is theoretically an "exact clone" of the ewe donating the genomic DNA, other factors prevent Dolly from being a complete duplicate. First, the genomic DNA may have incurred damage during the cloning procedure; thus Dolly may not be genetically equivalent to the donor cell ewe. Second, Dolly's genomic DNA was exposed to the maternally-contributed contents of the oocyte, which may fundamentally affect development.

The oocyte contents also include mitochondria, which contain their own actively expressed DNA. Finally, Dolly was exposed to a womb environment different from the womb environment in which the donor cell ewe developed. Thus, embryonic development depends upon these factors as well as the actual sequence of the genomic DNA, Dolly may be quite different from the donor cell ewe.

The cloning of Dolly raises a number of serious questions and concerns. The science used to produce Dolly is not well understood, and her status as a duplicate remains unclear due to the numerous influences upon her embryonic development. Dolly will need to be examined for any potential ill effects associated with the cloning procedure. The life span of her cells, her longevity as an animal, and her fertility should all be closely monitored. Finally, the future legal status of human cloning research needs to be resolved in an informed and timely manner.

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Chapter 14

In vitro Fertilization

1. Introduction

To understand assisted reproduction and how it can help infertile couples, it is important to understand how conception takes place naturally. For traditional conception to occur, the man must ejaculate his semen, the fluid containing the sperm, into the woman's vagina around the time of ovulation, when her ovary releases an egg. Ovulation is a complex event controlled by the pituitary gland, which is located at the base of the brain. The pituitary gland releases follicle-stimulating hormone (FSH), which stimulates follicles in one of the ovaries to begin growing. The follicle produces the hormone estrogen and contains a maturing egg. When an egg is mature, the pituitary gland sends a surge of luteinizing hormone (LH) that causes the follicle to rupture and release (ovulate) a mature egg (Figure 1). Following ovulation, the egg is picked up by one of the fallopian tubes. Because fertilization usually takes place inside the fallopian tube, the man's sperm must be capable of swimming through the vagina and cervical mucus, up the cervical canal into the uterus, and up into the fallopian tube, where it must penetrate the egg to fertilize it. The fertilized egg continues traveling to the uterus and implants in the uterine lining, where it continues to develop.

Solid arrows indicate path sperm must travel to reach the egg. Dotted arrow indicates path of egg. The fertilized egg continues traveling through the fallopian tube to the uterus.
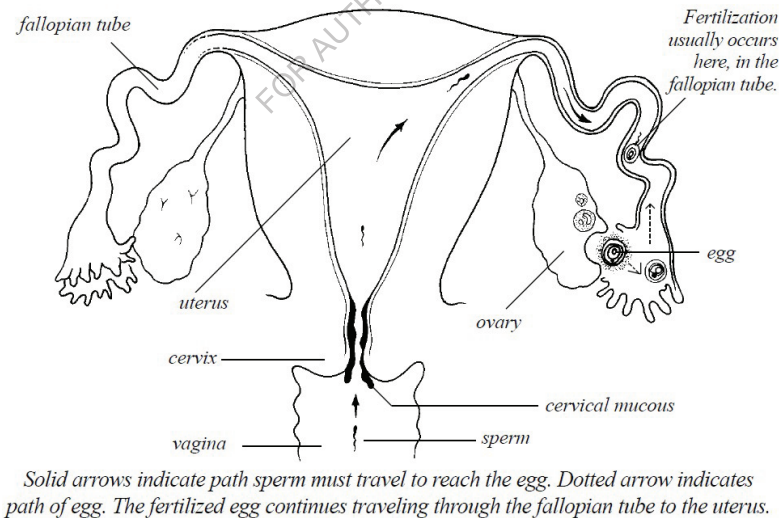
Figure 1 Reproduction Process

2. *In vitro* Fertilization (IVF)

There are many factors that can prevent the union of sperm and egg. Fortunately, assisted reproductive techniques such as IVF can help. IVF is a method of assisted reproduction in which a man's sperm and a woman's eggs are combined outside of the body in a laboratory dish. One or more fertilized eggs (embryos) may be transferred to the woman's uterus, where they may implant in the uterine lining and develop. Excess embryos may be cryopreserved (frozen) for future use. Initially, IVF was used to treat women with blocked, damaged, or absent fallopian tubes. Today, IVF is used to treat many causes of infertility, such as endometriosis and male factor, or when a couple's infertility is unexplained. The basic steps in an IVF treatment cycle are ovarian stimulation, egg retrieval, fertilization, embryo culture, and embryo transfer.

2.1 Ovarian Stimulation

During ovarian stimulation, also known as ovulation induction, medications or "fertility drugs," are used to stimulate multiple eggs to grow in the ovaries rather than the single egg that normally develops each month (Table 1). Multiple eggs are stimulated because some eggs will not fertilize or develop normally after fertilization. The maximum numbers of embryos transferred are based on the patient's age and other individual patient and embryo characteristics. Because each embryo has a probability of implantation and development, the number of embryos to be placed should be determined for each patient, taking into account the odds of achieving a pregnancy based on the number of embryos transferred weighed against the risk of multiple gestation (Table 1).

Table 1

Medications for Ovarian Stimulation
- clomiphene citrate (Clomid®, Serophene®)
- letrozole (Femara),
- follicle stimulating hormone (FSH) (FollistimTM, Gonal-F®, BravelleTM)
- human menopausal gonadotropins (hMG) (HumegonTM, RepronexTM, Menopur®)
- luteinizing hormone (LH) (Luveris®)

Medications for Oocyte Maturation
- human chorionic gonadotropin (hCG) (Profasi®, APL®, Pregnyl®, NovarelTM, Ovidrel®)

Medications to Prevent Premature Ovulation
- GnRH agonists (Lupron® and Synarel®)
- GnRH antagonists (Antagon®, Ganarelix® and Cetrotide®)

Clomiphene citrate is administered orally while the other medications listed are given by injection. Clomiphene citrate is less potent than injectable medications and is not as commonly used in ART cycles. There is no evidence that one injectable medication is superior to any other.

2.1.1 Medications for Inducing Ovulation

Timing is crucial in an IVF cycle. The ovaries are evaluated during treatment with vaginal ultrasound examinations to monitor the development of ovarian follicles (Figure 2). Blood samples may be drawn to measure response to ovarian stimulation medications. Normally, estrogen levels increase as the follicles develop, and progesterone levels are low until after ovulation.
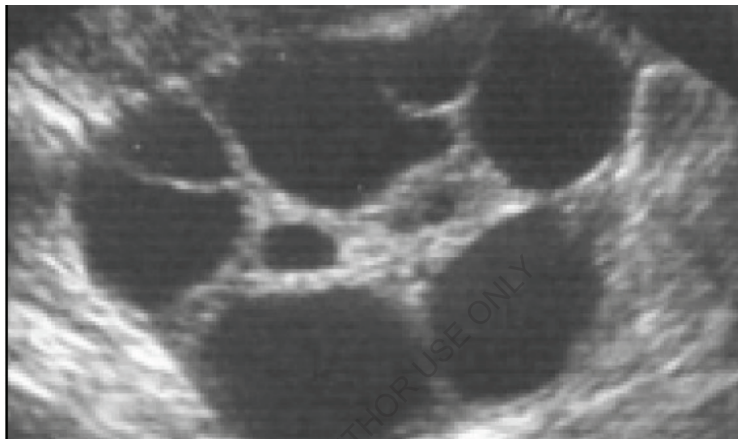
Figure 2 Ovarian follicles, stimulated by ovulation drugs, visible on ultrasound. The dark, circular areas are the follicles.

Using ultrasound examinations and blood testing, the physician can determine when the follicles are appropriate for egg retrieval. Generally, eight to 14 days is required. When the follicles are ready, hCG or other medications are given. The hCG replaces the woman's natural LH surge and causes the final stage of egg maturation so the eggs are capable of being fertilized. The eggs are retrieved before ovulation occurs, usually 34 to 36 hours after the hCG injection is given.

Up to 20% of cycles may be cancelled before egg retrieval. IVF cycles may be cancelled for a variety of reasons, usually due to an inadequate number of follicles developing. Cancellation rates due to low response to the ovulation drugs increase with age, especially after age 35. When cycles are cancelled due to a poor response, alternate drug strategies may be helpful to promote a better response in a future attempt. Occasionally, a cycle may be cancelled to reduce the risk of ovarian hyperstimulation syndrome (OHSS).

Treatment with a GnRH agonist or antagonist reduces the possibility of premature LH surges from the pituitary gland, and thereby reduces the risk of premature ovulation. However, LH surges and ovulation occur prematurely in a small percentage of ART cycles despite the use of these drugs. When this occurs, as it is unknown when the LH surges began

and eggs will mature, the cycle is usually cancelled. Collection of eggs from the peritoneal cavity after ovulation is not efficient.

2.1.2 Egg Retrieval

Egg retrieval is usually accomplished by transvaginal ultrasound aspiration, a minor surgical procedure that can be performed in the physician's office or an outpatient center. Some form of analgesia is generally administered. An ultrasound probe is inserted into the vagina to identify the follicles, and a needle is guided through the vagina and into the follicles (Figure 3). The eggs are aspirated (removed) from the follicles through the needle connected to a suction device. Removal of multiple eggs can usually be completed in less than 30 minutes.

Some women experience cramping on the day of the retrieval, but this sensation usually subsides by the next day. Feelings of fullness and/or pressure may last for several weeks following the procedure because the ovaries remain enlarged. In some circumstances, one or both ovaries may not be accessible by transvaginal ultrasound. Laparoscopy may then be used to retrieve the eggs using a small telescope placed in the umbilicus.
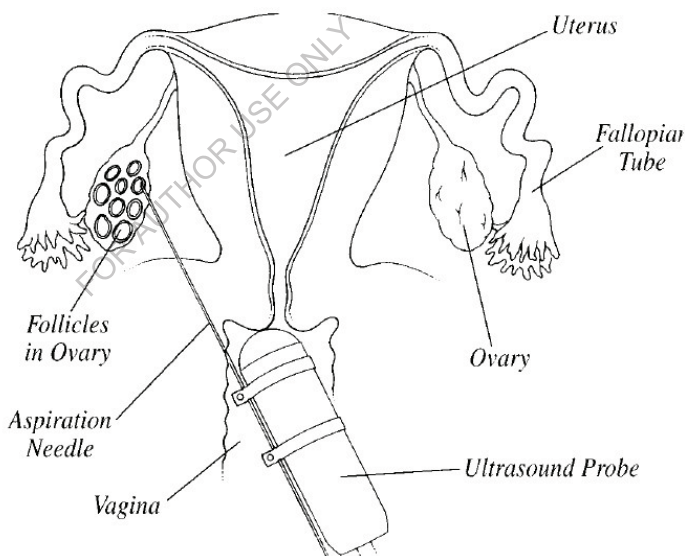
Figure 3 Egg retrieval is usually performed through the vagina with an ultrasound-guided needle.

2.1.3 Fertilization and Embryo Culture

After the eggs are retrieved, they are examined in the laboratory for maturity and quality. Mature eggs (Figure 4) are placed in an IVF culture medium and transferred to an incubator to await fertilization by the sperm.

Sperm is separated from semen usually obtained by ejaculation or in a special condom used during intercourse. Alternatively, sperm may be obtained from the testicle or vas deferens from men whose semen is void of sperm either due to an obstruction or lack of production.

Fertilization may be accomplished by insemination, where motile sperm are placed together with the oocytes and incubated overnight or by intracytoplasmic sperm injection (ICSI), where a single sperm is directly injected into each mature egg (Figure 5). In the United States, ICSI is performed in approximately 60% of ART cycles. ICSI is usually performed when there is a likelihood of reduced fertilization, i.e., poor semen quality, history of failed fertilization in a prior IVF cycle, etc.

Overall, pregnancy and delivery rates with ICSI are similar to the rates seen with traditional IVF. Genetic counseling is advisable before ICSI if inherited abnormalities are identified that may be passed from father to son.

Figure 4 A mature, unfertilized egg.

Visualization of two pronuclei the following day confirms fertilization of the egg. One pronuclei is derived from the egg and one from the sperm. Approximately 40% to 70% of the mature eggs will fertilize after insemination or ICSI. Lower rates may occur if the sperm and/or egg quality are poor. Occasionally, fertilization does not occur at all, even if ICSI was used.

Two days after the egg retrieval, the fertilized egg has divided to become a 2-to-4-cell embryo (Figure 6). By the third day, a normally developing embryo will contain approximately 6 to 10 cells. By the fifth day, a fluid cavity forms in the embryo, and the placenta and fetal tissues begin to separate. An embryo at this stage is called a blastocyst. Embryos may be transferred to the uterus at any time between one to six days after the egg retrieval. If successful development continues in the uterus, the embryo hatches from the surrounding zona pellucida and implants into the lining of the uterus approximately six to 10 days after the egg retrieval.

Larger tube that holds the egg still.

Small glass tube through which the sperm is injected into the egg.
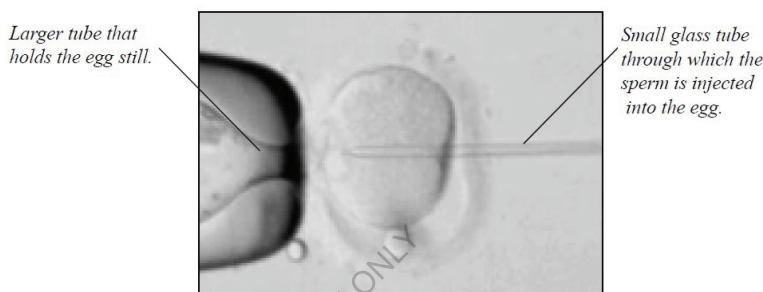
Figure 5 Intracytoplasmic sperm injection (ICSI), in which a sperm is injected directly into an egg to facilitate fertilization.

Figure 6 A fertilized egg that has divided once and is now a two-cell embryo.

2.2 Assisted hatching (AH)

Assisted hatching (AH) is a micromanipulation procedure in which a hole is made in the zona pellucida just before embryo transfer to facilitate hatching of the embryo. Although AH has not been demonstrated definitively to improve live birth rates, AH may be used for older women or couples who have failed prior IVF attempts. There is no clear benefit of AH to improve pregnancy or live birth rates in other groups of IVF patients.

2.3 Preimplantation genetic diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) is performed at some centres to screen for inherited diseases. In PGD, one or two cells are removed from the developing embryo and tested for a specific genetic disease. Embryos that do not have the gene associated with the disease are selected for transfer to the uterus. These procedures require specialized equipment and experience and IVF in a couple who may not need IVF to conceive.

The benefit of screening the embryos for disease in couples at risk and reducing the risk of having an affected child or terminating a pregnancy is prefereable to some couples. While PGD can reduce the likelihood of conceiving a pregnancy with an affected child, it cannot eliminate the risk. Confirmation with chorionic villus sampling (CVS), amniocentesis or other testing is still necessary.

2.4 Embryo Transfer

The next step in the IVF process is the embryo transfer. No anesthesia is necessary, although some women may wish to have a mild sedative. The physician identifies the cervix using a vaginal speculum. One or more embryos suspended in a drop of culture medium are drawn into a transfer catheter, a long, thin sterile tube with a syringe on one end. The physician gently guides the tip of the transfer catheter through the cervix and places the fluid containing the embryos into the uterine cavity (Figure 7). The procedure is usually painless, although some women experience mild cramping.

ASRM issues guidelines regarding determination of how many embryos should be considered for transfer. The number of embryos transferred is largely based on the age of the woman or oocyte donor undergoing IVF retrieval.

These guidelines were created to help maintain the high success rates of IVF centers while decreasing the number of higher order multiple pregnancies (triplets and higher). The reproductive endocrinologist or embryologist will discuss this with the patient before the transfer.

2.5 Cryopreservation

Extra embryos remaining after the embryo transfer may be cryopreserved (frozen) for future transfer. Cryopreservation makes future ART cycles simpler, less expensive, and less invasive than the initial IVF cycle, because the woman does not require ovarian stimulation or egg retrieval. Once frozen, embryos may be stored for several years. However, not all embryos survive the freezing and thawing process, and the live birth rate is lower with cryopreserved embryo transfer. Couples should decide if they are going to cryopreserve extra embryos before undergoing IVF.
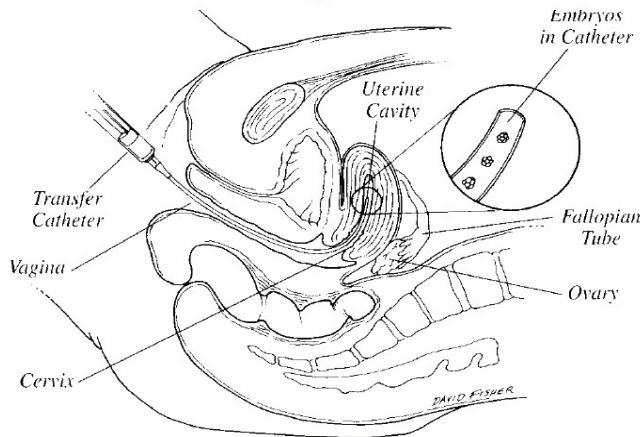
Figure 7 Embryo transfer is performed through the cervix.

2.6 Variations of IVF

Gamete intrafallopian transfer (GIFT) is similar to IVF, but the gametes (egg and sperm) are transferred to the woman's fallopian tubes rather than her uterus, and fertilization takes place in the tubes rather than in the lab. Another difference is that laparoscopy, a surgical procedure, is necessary to transfer the sperm and egg to the tubes. GIFT is an option only for women who have normal fallopian tubes. Some couples may consider GIFT for religious reasons because eggs are not fertilized outside the body. One limitation of GIFT is that fertilization cannot be confirmed as with IVF. Today, GIFT comprises less than 1% of ART procedures performed in the United States.

Another ART procedure is zygote intrafallopian transfer (ZIFT). This technique differs from GIFT in that fertilization takes place in the lab rather than the fallopian tube, but is similar in that the fertilized egg is transferred to the tube rather than the uterus. This procedure also requires a laparoscopy. Today, ZIFT comprises less than 1.5% of ART cases performed in the United States.

2.7 Success Rates

The most recent rates for individual IVF programs in the United States are available on the Internet from the Society for Assisted Reproductive Technology (SART) at www.sart.org and from the Centers for Disease Control and Prevention: www.cdc.gov/art. Although this information is readily available, the results should be interpreted carefully. The success rates of an IVF center depend upon a number of factors, and a comparison of clinic success rates is not meaningful because patient characteristics and treatment approaches vary from clinic to clinic. For example, the type of patients accepted into the program and the numbers of embryos transferred per cycle affects the program's statistics.

Statistics calculated on small numbers of cycles may not be accurate. An IVF center's rates may change dramatically over time, and the compiled statistics may not represent a program's current success.

It is also important to understand the definitions of pregnancy rates and live birth rates. For example, a pregnancy rate of 40% does not mean that 40% of women took babies home. Pregnancy does not always result in live birth. A biochemical pregnancy is a pregnancy confirmed by blood or urine tests but not visible on ultrasound, because the pregnancy stops developing before it is far enough along to be seen on ultrasound. A clinical pregnancy is one in which the pregnancy is seen with ultrasound, but stops developing sometime afterwards. Therefore, when comparing the "pregnancy" rates of different clinics, it is important to know which type of pregnancy is being compared.

Most couples are more concerned with a clinic's live birth rate, which is the probability of delivering a live baby per IVF cycle started. Pregnancy rates, and more importantly live birth rates, are influenced by a number of factors, especially the woman's age.

3. Donor Sperm, Eggs and Embros

IVF may be done with a couple's own eggs and sperm or with donor eggs, sperm, or embryos. A couple may choose to use a donor if there is a problem with their own sperm or eggs, or if they have a genetic disease that could be passed on to a child. Donors may be known or anonymous. In most cases, donor sperm is obtained from a sperm bank. Sperm donors undergo extensive medical and genetic screening, as well as testing for infectious diseases. The sperm are frozen and quarantined for six months, the donor is re-tested for infectious diseases including the AIDS virus, and sperm are only released for use if all tests are negative. Donor sperm may be used for insemination or in an ART cycle.

Overall, the use of frozen sperm rather than fresh sperm does not lower success rates. Donor eggs are an option for women with a uterus who are unlikely or unable to conceive with their own eggs. Egg donors undergo the same medical and genetic screening as sperm donors, although it is not currently possible to freeze and quarantine eggs like sperm. The egg donor may be chosen by the infertile couple or the ART program. Egg donors assume more risk and inconvenience than sperm donors. In the United States, egg donors selected by ART programs generally receive monetary compensation for their participation. Egg donation is more complex than sperm donation and is done as part of an IVF procedure. The egg donor must undergo ovarian stimulation and egg retrieval. During this time, the recipient (the woman who will receive the eggs after they are fertilized) receives hormone medications to prepare her uterus for implantation. After the retrieval, the donor's eggs are fertilized by sperm from the recipient's partner and transferred to the recipient's uterus. The recipient will not be genetically related to the child, but she will carry the pregnancy and give birth.

Egg donation is expensive because donor selection, screening, and treatment add additional costs to the IVF procedure. However, the relatively high live birth rate for egg donation, approximately 50% nationally, provides many couples with their best chance for success. Overall, donor eggs are used in nearly 10% of all ART cycles in the United States.

In some cases, when both the man and woman are infertile, both donor sperm and eggs have been used. Donor embryos may also be used in these cases. Some IVF programs allow couples to donate their unused frozen embryos to other infertile couples. Appropriate

screening of the individuals whose genetic embryos were used to create these embryos should adhere to federal and state guidelines.

The use of donor sperm, eggs, or embryos is a complicated issue that has lifelong implications. Talking with a trained counselor who understands donor issues can be very helpful in the decision-making process. Many programs have a mental health professional on staff or the physician may recommend one. If a couple knows the donor, their physician may suggest that both the couple and the donor speak with a counselor and an attorney. Some states require and most IVF centers recommend an attorney to file paperwork for the couple with the court when donor gametes or embryos are used.

4. Surrogacy/Gestational Carrier

A pregnancy may be carried by the egg donor (traditional surrogate) or by another woman who has no genetic relationship to the baby (gestational carrier). If the embryo is to be carried by a surrogate, pregnancy may be achieved through insemination alone or through ART. The surrogate will be biologically related to the child. If the embryo is to be carried by a gestational carrier, the eggs are removed from the infertile woman, fertilized with her partner's sperm, and transferred into the gestational carrier's uterus. The gestational carrier will not be genetically related to the child. All parties benefit from psychological and legal counseling before pursuing surrogacy or a gestational carrier.

5. Steps in the IVF-ET Process

a. Day 0--Day of Egg Retrieval

When eggs are brought to the laboratory, they are removed from the follicular fluid, washed and placed in a nutrient solution (medium) in an incubator. The sperm is also washed and processed in such a way that many of the dead and abnormal sperm are removed, and the motile sperm are concentrated. The sperm is incubated until the afternoon. If the sperm appear normal, and there is no suggestion from the patient's history that fertilization might be a problem (e.g., unexplained infertility), the sperm are placed near the eggs, and they are incubated overnight.

If there is a history of a male factor, unexplained infertility, poor fertilization in a prior IVF cycle, or a poor sample on this particular day, the sperm are physically injected into each egg using Intracytoplasmic Sperm Injection (ICSI).

b. Day 1--Fertilization Check

Early in the morning, the eggs are checked for signs of fertilization. At this time, normally fertilized eggs will have 2 nuclei in the center, one from the egg and one from the sperm. (as shown below)

Any number of nuclei greater or lesser than 2 indicates abnormal fertilization. Normally fertilized eggs are now considered to be embryos, and are separated from the unfertilized and abnormally fertilized eggs.

Normally at this time, the decision is made about whether the day of embryo transfer will be on day 3 or 5. This decision is based on the number of embryos there are and their

quality, the wife's age, and any history from prior IVF cycles. If the decision is not clear-cut at this time, it will be made on the morning of Day 2.

Late in the afternoon the embryos are checked for development. Those embryos that have begun to grow are separated from those that have not, as they have a better chance of continuing development.

c. Day 2-- Development Check

Early in the morning, the embryos are again checked for development. At this point in time, embryos should be between 2 and 4 cells. Embryos that have not divided by this time are separated from the others as their prognosis is poor.

d. Day 3--Assisted Hatching and/or Transfer

Again early in the morning, the embryos are checked for development and are photographed.

Day 3 Transfer

For patients having a day 3 transfer, the best quality embryos are selected. Ideally, the best quality embryos will have 8 cells. (as shown below)

Anywhere from 3-5 embryos will be transferred, depending on the women's age, and the quality of the embryos.

Some patients will have their embryos treated with Assisted Hatching on this day. Patients who are 36 and older, who have a high FSH level, who require large amounts of medication to get to egg retrieval, whose eggs have thick coat of protein surrounding them, or who have not become pregnant on a prior IVF cycle, are candidates for this procedure. In this procedure, the best embryos are chosen, and a solution is blown against the protein coat of the embryo and a small hole is created. This will help the embryo to come in contact with the lining of the uterus.

Embryos continue incubation. They will begin a process called compaction. (as shown below)

Young patients may often have compacted embryos on day 3.

In any group of embryos, it is to be expected that some will develop slowly, and a few may stop development altogether.

e. Day 4

In the morning, embryos are evaluated and photographed. At this point in time, embryos that have continued development should be at the blastocyst stage. (as shown below)

They now have 60-100 cells, and an inner cavity that is filled with fluid. Depending on the woman's age and quality of the embryos 2-3 will be transferred. If there are no embryos that have developed to blastocyst, morula stag embryos (the stage just before blastocyst) will be transferred. Three to four morulae may be transferred.

f. Day 5--Embryo Transfer and/or Cryopreservation

If embryos are to be transferred to a patient, the best embryos are chosen for transfer, and any extra blastocysts may be frozen.

Young patients, and patients who have had Assisted Hatching, may have blastocysts that have begun the hatching process.

g. Day 6--Cryopreservation

Any embryos that have continued development in culture and have developed to the blastocyst stage may be frozen. All other embryos are discarded.

6. Risks of ART

The medical risks of ART depend upon each specific step of the procedure. The following are some of the primary risks of ART procedures Ovarian stimulation carries with it a risk of hyperstimulation, where the ovaries become swollen and painful. Fluid may accumulate in the abdominal cavity and chest, and the patient may feel bloated, nauseated, and experience vomiting or lack of appetite. Up to 30% of patients undergoing ovarian stimulation have a mild case of ovarian hyperstimulation syndrome (OHSS) that can be managed with over-the-counter painkillers and a reduction in activity. In moderate OHSS, patients develop or accumulate fluid within the abdominal cavity, and gastrointestinal symptoms may occur. These women are monitored closely, but generally do very well with simple outpatient management. The condition tends to resolve without intervention unless pregnancy occurs, in which ase recovery may be delayed for several weeks. Up to 2% of patients develop severe OHSS characterized by excessive weight gain, fluid accumulation in the abdomen and chest, electrolyte abnormalities, over-concentration of the blood, and rarely the development of blood clots, kidney failure or death. It may be medically necessary to drain fluid from the abdomen with a needle if breathing becomes difficult. Patients with severe OHSS require hospitalization until the symptoms improve. If pregnancy occurs, OHSS can worsen. Occasionally, termination of pregnancy must be considered in the most severe cases. Although initial reports suggested that women who use fertility drugs have an increased risk for ovarian cancer, numerous recent studies support the conclusion that fertility drugs are not linked to ovarian cancer. Nevertheless, there is still uncertainty whether a risk exists and research continues to address this question.

An annual gynecologic visit is recommended for all women, with examination of the ovaries, regardless of prior use of ovulation medications. There are risks related to the egg retrieval procedure. Laparoscopy carries with it the risks of any surgery that requires anesthesia. Removing eggs through an aspirating needle entails a slight risk of bleeding, infection, and damage to the bowel, bladder, or a blood vessel. This is true whether the physician uses laparoscopy or ultrasound to guide the needle. Less than one patient in 1,000 will require major surgery to repair damage from complications of the egg retrieval procedure. In rare cases, infection may occur from the retrieval or embryo transfer.

The chance of multiple pregnancies is increased in all assisted reproductive technologies when more than one embryo is transferred. Although some would consider twins a happy result, there are many problems associated with multiple births; and problems become progressively more severe and common with triplets and each additional fetus thereafter. Women carrying a multiple pregnancy may need to spend weeks or even months in bed or in the hospital in an attempt to delay premature delivery. The risk of premature

delivery in multiple pregnancies is high, and babies may be born too early to survive. Premature babies require prolonged and intensive care and risk lifelong handicaps due to premature birth.

Some couples may consider multifetal pregnancy reduction to decrease the risks due to multiple pregnancy, but this is likely to be a difficult decision. Data also suggest that IVF conceptions, even singletons, have a slightly increased risk of premature delivery or low birth weight. First trimester bleeding may signal a possible miscarriage or ectopic pregnancy. If bleeding or pain (before 13 weeks) occurs, a medical evaluation is needed to determine the cause. Some evidence suggests that early bleeding is more common in women who undergo IVF and GIFT and is not associated with the same poor prognosis as it is in women who conceive spontaneously.

Miscarriage may occur after ART, even after ultrasound identifies a pregnancy in the uterus. Miscarriage occurs after ultrasound in nearly 15% of women younger than age 35, in 25% at age 40, and in 35% at age 42 following ART procedures. In addition, there is approximately a 5% chance of ectopic pregnancy with ART. It is not clear whether the risk of birth defects is increased with IVF. Most studies do not show an increased risk, but several studies do. Research is ongoing to determine the magnitude, if any, of this risk. Furthermore, when ICSI is used in cases of severe male factor infertility, a genetic cause of male infertility may be passed on to the offspring.

Assisted reproductive technologies involve a significant physical, financial, and emotional commitment on the part of the couple. Psychological stress is common; and some couples describe the experience as an emotional roller coaster. The treatments are involved and costly. Patients have high expectations, yet failure is common in any given cycle. Couples may feel frustrated, angry, isolated, and resentful. At times, frustration can lead to depression and feelings of low self-esteem, especially in the immediate period following a failed ART attempt. The support of friends and family members is very important at this time. Couples are encouraged to consider psychological counseling as an additional means of support and stress management. Many programs have a mental health professional on staff to help couples deal with the grief, tension, or anxieties associated with infertility and its treatment.

7. Preparation for ART

Preliminary preparation for an ART procedure may be as important as the procedure itself. Testing for ovarian reserve may be recommended to predict how the ovaries will respond to fertility medication. The chance of success may be poor, for example, if tests demonstrate diminished ovarian reserve or fertility potential. Ovarian reserve may be determined by measuring FSH and estradiol levels on the second or third day of a menstrual cycle, performing a clomiphene challenge test, or counting the number of beginning follicles in the ovary (antral follicle count). An elevated FSH and/or estradiol level is associated with reduced pregnancy rates, especially in women over the age of 35 years. Uterine cavity abnormalities such as fibroids, polyps, or a septum may need to be corrected before IVF or GIFT. A hydrosalpinx, a fluidfilled, blocked fallopian tube, reduces IVF success. Some physicians advise clipping or removing the affected tube before IVF. For more information, see the ASRM patient fact sheet titled, Hydrosalpinx.

Semen is tested before ART. If semen abnormalities are identified, consultation with a specialist in male infertility should determine if there are correctable problems or underlying health concerns. For example, genetic abnormalities in the Y chromosome have been linked to some cases of male infertility; and men born without a vas deferens, a tube that transports sperm from the testicle, are often carriers of a gene that causes cystic fibrosis. In these circumstances, genetic testing may be advisable.

Major advances have been made in the treatment of male infertility, and IVF may help some men who were previously considered sterile. Detailed consultation with a specialist in male infertility is essential. When sperm cannot be collected by masturbation, electroejaculation (EEJ), microepididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), or testicular sperm extraction (TESE) may be effective methods to collect sperm for IVF. EEJ may be an effective way to collect sperm from men with spinal cord injuries. During EEJ, electrical impulses from a probe placed in the rectum near the prostate often accomplish ejaculation. MESA can be performed to recover sperm after vasectomy or failed vasectomy reversal and in some men with absence of the vas deferens. TESE involves testicular biopsy and recovery of sperm directly from testicular tissue and may be performed in an office setting with local anesthesia. Sperm obtained by these methods may be frozen, stored, and thawed later for ART.

Lifestyle issues should be addressed before ART. Smoking, for example, may lower a woman's chance of success by as much as 50%. All medications, including over-the-counter supplements, should be reviewed because some may have detrimental effects. Alcohol and recreational drugs may be harmful, and excessive caffeine consumption should be avoided. Because folic acid taken before pregnancy reduces the risk of neural tube defects such as spina bifida, women should take prenatal vitamins containing at least 400 micrograms of folic acid before beginning an ART cycle. A complete exam and Pap smear may identify problems that should be treated before pregnancy.

A detailed examination of ART insurance benefits is helpful. Even if ART is excluded from a policy, coverage may be available for some aspects of these procedures. Couples should consult their companies' benefits director in advance, because options such as a medical savings account may be available. It is also important to determine the costs for the ART treatment cycle. Keep in mind that fees for initial consultation, screening tests, medications, and special procedures such as ICSI and cryopreservation may not be included in the estimate. Other expenses to consider include travel, lodging, and time missed from work.

8. Selecting an ART Program

When selecting an ART program, information is crucial. Important points for consideration include the qualifications and experience of personnel, types of patients being treated, support services available, cost, convenience, live birth rates per ART cycle started, and multiple pregnancy rates. Older programs have established live birth rates based on years of experience. Small and new programs may still be determining their live birth rates, although their personnel may be equally well qualified.

Every couple wants to use the most successful ART program, but many factors contribute to the overall success of a program. For example, some clinics may be willing to accept patients with a low chance of success. A clinic may specialize in certain types of

infertility treatment. Costs may vary between programs. A couple may prefer a program based on interpersonal interactions with the ART team, or may feel more confident in the recommended treatment plan. Consequently, it is not appropriate to compare programs based only on the published pregnancy rates.

Credibility is important too. Does the program adhere to the guidelines set forth by the American Society for Reproductive Medicine (ASRM)? Is the program a member of SART, a society affiliated with the ASRM? Is the IVF lab accredited by the College of American Pathologists or by the Joint Commission? These organizations require ART programs to have personnel on their staffs that have been trained in reproductive endocrinology, laparoscopic surgery, sonography, hormone measurement, tissue culture technique, and sperm/egg interaction. Are the physicians board certified in reproductive endocrinology and infertility? Does the program report its results to SART/CDC? The compiled results are published in Fertility and Sterility, the ASRM journal, and results are available on the SART Web site at www.sart.org and the CDC's Web site at: www.cdc.gov/art. The above considerations and answers to the following questions, which may be asked of each program, will help you make an informed decision when choosing an ART program.

8.1 Cost and Convenience

- What pre-cycle screening tests are required, how much do they cost, and will my insurance provide coverage for these tests?
- How much does the ART procedure cost, including drugs per treatment cycle?
- Do I pay in advance? How much? What are the methods of payment?
- If applicable, will you submit any bills to my insurance company?
- How much do I pay if my treatment cycle is canceled before egg recovery? Before embryo transfer?
- What are the costs for embryo freezing, storage, and transfer?
- How much work will I miss? How much will my partner miss?
- Do you help arrange (low-cost) lodging, if needed?

8.2 Details about the Program

- Is the program a member of the Society for Assisted Reproductive Technology?
- Does the program meet and follow ASRM/SART guidelines?
- Does the program report its results to SART/CDC?
- How many physicians will be involved in my care?
- Are one or more physicians board certified in reproductive endocrinology?
- To what degree can my own physician participate in my care?
- What types of counseling and support services are available?
- Whom do I call day or night if I have a problem?
- Do you freeze embryos (cryopreservation)?
- Is donor sperm available in your program? Donor eggs? Donor embryos?
- Do you have an age or basal FSH cut-off?
- Do you perform ICSI? If so, when? What is the cost?
- Do you perform assisted hatching? If so, when? What is the cost?
- How many eggs/embryos would be transferred in my case?
- Who makes the final decision to cancel the cycle if my response to stimulation is sub-optimal?

9. Success of the Program

SART is a very good source of information from which to to obtain ART outcomes for each member program in the United States. This information may be a year old, so it is important to find out if there have been any significant changes in the program because the most recent report including:

- Personnel changes
- Changes in the approach to ovarian stimulation, egg retrieval, embryo culture, or embryo transfer.
- Change in the number of cycles
- Change in the miscarriage rate, live birth rate per cycle started, or the multiple pregnancy rate.

If a program cites a live birth rate for each procedure, be sure that the program representative ounts twins as one successful pregnancy, not two. When discussing recent ART program outcomes, keep in mind that the live birth rate may vary depending on the denominator used - i.e., per cycle started, per retrieval, or per embryo transfer. For example, live birth rates per egg retrieval do not consider cancelled cycles, and rates based per embryo transfer do not include cancelled cycles or fertilization failures. Therefore, live birth rates per cycle are higher per egg retrieval and are highest per embryo transfer.

10. When to end treatment

Studies indicate that the chance for pregnancy in consecutive IVF cycles remains similar in up to four cycles. However, many other factors should be considered when determining the appropriate endpoint in therapy, including financial and psychological reserves. Members of the IVF team can help couples decide when to stop treatment and discuss other options such as egg and/or sperm donation or adoption, if appropriate. The physician, support groups, and other couples undergoing infertility treatment can provide valuable support and guidance.

11. Conclusion

It is a privilege for any scientist or clinician to have access to the earliest stages of human development through culturing gametes and embryos *in vitro*. And, as such, it requires a high degree of ethical responsibility to provide as safe and optimal an environment as possible for these microscopic changes. While much has been done to maximize IVF pregnancy rates over the last two decades, it nevertheless remains to improve individual embryo selection to the point where we can routinely transfer only one embryo at a time, while being able to successfully and consistently freeze all surplus embryos of sufficient quality for later use in attempting pregnancy.

In vitro fertilization in India

In vitro fertilization (IVF) and embryo transfer were successfully introduced to treat human infertility in 1978 and the world's first test-tube baby, Louise Brown, was born in the UK on 28 July of that year. Exactly 67 days later, Subhash Mukerjee announced the birth of the world's second test-tube baby, Kanupriya, alias Durga, on 3 October 1978 in Calcutta. Both these announcements were received with skepticism and the scientists respons ible for these births were severely criticized. Nevertheless, the British team carried on with their work

and produced several more test-tube babies. In marked contrast, the Government of West Bengal proscribed Mukerjee from carrying out further work in this area and he was transferred to an eye hospital that did not have any facilities to enable him pursue his work.

Mukerjee could not publish all the details of his work. He was asked by the Government to submit details of his work, which he did. He also stated that he wished to carry out further work so that he could validate and standardize the various procedures he had used in his first and only success. His being prohibited to carry out further work by the Government of West Bengal and his untimely death left big lacunae in our understanding the rationale behind the techniques he used. However, in the report he submitted to the Government of West Bengal, which was signed by all the three investigators that were involved in carrying out this procedure, some details were given. These details clearly indicate that Mukerjee's techniques were different from those used by the British team. IVF in India would have continued to languish but for the support from the ICMR to support IVF-ET that led to the birth of Harsha in Mumbai in 1986. Several other clinics soon followed suit and today there are reportedly over 200 clinics claiming to offer IVF all over India.

A million test-tube babies have reportedly been born all over the world. IVF has turned out to be a major scientific achievement of mankind during the last century. It has not only opened out novel ways of treating infertility involving third and sometimes fourth party parenting a child in a tandem manner, but also advanced our understanding of basic biology and pathology of human reproduction. With new developments occurring in the potential use of embryonic stem cells in the development of biotherapeutics, IVF is the only way to obtain pluripotential embryonic stem cells.

In view of these vast developments in the field of IVF it is only proper that the pioneers in the field are remembered and honoured. Britain celebrated the birth of Louise Brown with much fanfare on 28 July this year. The Indian Council of Medical Research in collaboration with the Inter Academy Biomedical Science Forum and Hope Infertility Clinic commemorated the silver jubilee of IVF in India, at a simple function held in the premises of the Indian Academy of Sciences, Bangalore. On behalf of the Director General of the Indian Council of Medical Research, Vasantha Muthuswamy, felicitated Kanupriya, Sunit Mukerjee, the only surviving member of Mukerjee's team, and C. P. Puri, National Institute for Research in Reproductive Health, Mumbai for his personal contribution to the endocrine evaluation and monitoring of controlled ovarian hyper stimulation of women recruited for IVF, which was the cornerstone for the successful pregnancy to occur after IVF and embryo transfer. This was followed by a symposium on Spin-offs from Medically Assisted Reproductive Techniques. This meeting was aimed at informing the lay public as well as clinical faculty and scientists on some of the issues that have arisen out of greater understanding of human reproductive processes through medically assisted reproductive techniques. Little known facts about male infertility were covered by N. Pandiyan (Andrology and Reproductive Sciences, Apollo Hospitals, Chennai). Infertility is a personal tragedy in the lives of many couples.

Male factors are equally responsible for infertility as are those of the female. Much has been written about the causes of male infertility. Sexual dysfunction, ignorance of sexual practices like using lubricants during intercourse also contribute to male infertility. Semen analysis remains the single most important test for the evaluation of an infertile male, despite all its limitations.

Computer Assisted Semen Analysis (CASA) has not replaced conventional semen analysis. Men with abnormal semen picture would require further investigations like serum FSH, scrotal Doppler and genetic studies. Serum LH, prolactin and testosterone estimations are required only in men with sexual dysfunction and loss of libido. Sperm function studies are of limited clinical value. Recent data indicate that *in vivo* spermatozoa movements are not random movements but are directed by chemotaxis and thermotaxis.

The introduction of Intra Uterine Insemination (IUI), IVF and Intra Cytoplasmic Sperm Injection (ICSI) have all proven to be of immense value in the management of male infertility. However these new techniques have not eliminated the problem of male infertility as was claimed in the mid and late nineties. The problem of male infertility still remains. Cloning, or haploidization or stem cell therapy may be the only answer for biological fatherhood for many of these men. 'It is all in the environment: Incidence of male infertility varies between regions in infertile couples' was presented by Rajvi Mehta (Hope Infertility Clinic, Bangalore). Nearly 50% of infertility is related to reproductive defects or disorders in the male; the rest is related to the female. There has been growing evidence to suggest that in some regions the incidence of male infertility amongst infertile couples varies between regions. Such evidence has been accruing from different parts of the world and between different Indian cities.

A preliminary study in India shows that significant differences occur both in semen quality and sperm concentrations in fertile couples. There is reason to believe that environmental factors play an important role in this manifestation. There is a need to carry out a national study of environmental factors affecting male fertility. 'It is all in our genes: Genomics of male infertility' was presented by K. Kucheria, N. P. Gupta, and Rima Dada (All India Institute of Medical Sciences, New Delhi). Ten to 25% couples find difficulty to procreate. Micro deletion of the long arm of the Y chromosome is associated with spermatogenic failure and has been used to define three regions on Yq (AZFa, AZFb and AZFc) which are critical for spermatogenesis. These 3 loci act at different stages of germ cell development and deletion of each locus results in a characteristic phenotype. Deletion of AZFa, AZFb and AZFc results in Sertoli Cell Only (SCO) syndrome, maturation arrest and hypospermatogenesis respectively. One hundred and seventy five infertile males with idiopathic oligozoospermia and azoospermia were included in one study. Cytogenetic and semen analysis was done in each case. Testicular Fine Needle aspiration Cytology was collected whenever possible. Of the 175 cases, 22 were identified as Klinefelter Syndrome (KFS), 14 cases were mosaic KFS and 6 cases were variant KFS.

Various factors – genetic, epigenetic and environmental – modulate the effect of these genes. Thus, in a large number of idiopathic cases of male infertility there is a genetic basis. Therefore detection of Yq micro deletions encompassing the AZF loci determines the prognosis and management of these infertile cases. 'The dawn of a new era in biotherapeutics: Embryonic stem cells for organ repair or replacement' was presented by Satish Totey (Reliance Life Sciences, Mumbai). There has been much hope and hype associated with embryonic stem cells with reference to their possible use in futuristic biotherapies and regenerative medicine. However, as in the case of earlier hopes and even claims in areas such as use of monoclonal antibodies, gene therapy, etc. for treatment of diseases, there is as yet no clear evidence to support the concept of embryonic stem cells being taken up in the near future as biotherapeutic agents. This is because several basic questions need to be answered.

Some of these questions pertain to:

1. Immuno-rejection of stem cells when transferred to another individual.
2. Identifying cells signals that drive the developmental course of undifferentiated stem cells.
3. Growing stem cells in medium free from animal products to avoid zoonoses.

'Opportunities offered by the government of India for establishing co-operative research associations and assistance through program aimed at technological self reliance (PASTER)' was presented by T. C. Anand Kumar (Hope Infertility Clinic, Bangalore). Research in infertility, to understand some of the unexplained conditions of infertility, developing new and better forms of treatment, attaining selfreliance in products used in the treatment of infertility requires substantial inputs of technical talents as well as funding. Most of infertility treatment lies in the private sector. CSIR has a scheme that supports private industry that is of a cooperative nature. Establishing a collaborative, co-operative research facility in infertility research, which is a multicrore business/industry, may be a good way of attaining self-sufficiency in this industry.

In vitro fertilization techniques are mainly offered by the private sector, which is very heavily dependent on imported drugs, equipment and devices including disposable plastic ware used in *in vitro* culture. Consequently, the cost of IVF is extremely high in India and unaffordable to many. Research into aspects of human reproduction is almost nonexistent in the private sector and there is hardly any scientific paper that emerges from Indian laboratories or clinics practising IVF. In the symposium, collaboration between the private and pubic sector funding agencies was stressed. Examples were given of the programmes available under the Department of Scientific and Industrial Research such as the Program Aimed at Technological Self-Reliance (PATSER) and Co-operative Research Associations that enable the establishment of research centres as a collaborative effort between the private sector and Government funding agencies. This aspect was appreciated by the participants who endorsed the view that similar collaborations must be established between private IVF clinics and even the ICMR to address issues that are of common interest and are aimed at improving patient care and more importantly, providing indigenous substitutes for imported equipment, supplies and drugs.

Chapter 15
Gene therapy

1. Introduction

Genes are made of DNA - the code of life. They are made of a sequence of chemicals with the initials A, C, G and T, just like the alphabet makes words and sentences which can be turned into instructions. Everyone inherits genes from their parents and passes them on in turn to their children. Every person's genes are different, and the changes in sequence determine the inherited differences between each of us. Some changes, usually in a single gene, may cause serious diseases (such as Cystic Fibrosis, Muscular Dystrophy or Thalassemia). More often, gene variants interact with the environment to predispose some people to Cancer, or Heart disease, or other common ailments. Today doctors can look at a person's DNA using very sensitive new techniques which use as little as one hair root or drop of blood.

Gene therapy involves the introduction of genetic material into a cell to treat disease. Many of the conditions treated in this way are genetic diseases that occur when genes malfunction. A common approach in gene therapy is to identify a malfunctioning gene and supply the patient with functioning copies of that gene. Other approaches include switching specific genes on or off, introducing genes to kill cancer cells, to suppress tumours by inhibiting the blood supply, or to stimulate the immune system to attack certain types of cells. Whichever approach is used, the aim of gene therapy is to introduce therapeutic material into the target cells, for this to become active inside the patient and exert the intended therapeutic effect. At present, gene therapy is still at the clinical research stage. The UK Clinical Trials Regulations 2004 prohibit gene therapy on reproductive (germ line) cells; it can only be carried out on non-reproductive (somatic) cells. Germ line gene therapy can potentially cause changes in a patient, including harmful effects that could be passed on to future generations. It is therefore currently considered unacceptable for both ethical and safety reasons. Gene therapy is 'the use of genes as medicine'. It involves the transfer of a therapeutic or working gene copy into specific cells of an individual to repair a faulty gene copy. Thus it may be used to replace a faulty gene, or to introduce a new gene whose function is to cure or to favourably modify the clinical course of a condition. The scope of this new approach to the treatment of a condition is broad, with potential in the treatment of many genetic conditions, some forms of cancer and certain viral infections such as AIDS.

Gene therapy remains an experimental discipline however and much research remains to be done before this approach to the treatment of certain conditions will realise its full potential.

2. Somatic Gene Therapy

Our cells are divided into two groups, the somatic cells which make up the working parts of the body, and the germ cells (or sex cells: sperm in men and eggs in women) which pass on genetic material to our children. Every normal somatic cell contains the same coded DNA instructions, even if only some of them are used. Different ones will be active in different parts of the body. If a body cell is modified by Somatic Gene Therapy it will only

change cells in the body of the person being treated, and not be passed on to children. Somatic gene therapy can be targeted to, for example, the liver, blood or lungs, to correct a medical problem which exists and which can be treated by the gene or its protein product, such as an inherited disease or cancer.

2.1 Application to Somatic Cell Therapy

So far as we know, there has been no committee anywhere that has recommended outright prohibitions of all somatic cell gene therapy. The excellent 1982 Report "Splicing Life" (United States Presidential Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research) laid the groundwork for world-wide recognition that the ethical problems presented by such therapies are not fundamentally different from those presented by other research techniques. All members of the Subcommittee concur.

Contrary arguments, we believe, would have to be based on claims that:

a) there is an impermissible high chance of accidents causing large destruction to persons, property or the environment that will ultimately occur through accidental release of viral or other materials;

b) accidents in somatic cell therapy sooner or later will result in the alteration of germline cells; or,

c) the creation of somatic therapies puts us on the slippery slope to wrongful germline interventions, slope which we cannot hold.

The first argument has echoes in debates about the construction of nuclear power plants. We believe the premise wrong, even on the assumption accidents may happen. Furthermore, to accept the argument would imply the impermissibility of the full range of genetic technologies, technologies that will have major impact in world economic life. The prohibitions that would flow from accepting this risk argument are not limited (if it be thought a limit) to treatments of ill people.

As to the second argument, researchers cannot rule out or depreciate the prospect that some mishap may alter someone's germline cells, particularly as techniques to administer gene insertion *in vivo* are developed. Is the possible accidental alteration of a germline cell a basis for total prohibition of all somatic cell gene therapy? We think not.

Risks may and must be minimized by maintaining a constant focus on safety. But more important, the human genome is by no means stable. It constantly changes through mutation, including many mutations that result from human activities. Most mutations, of course, are selected against. They fail to survive. The same would be true with these kinds of accidents.

The slippery slope argument, by contrast, is itself an argument without stopping point. It would lead to bans on airplanes because they might be used to drop bombs (Sass, 1988). If people believe maintaining sharp boundaries against germline intervention is important, we think social, legal and professional standards will hold the line adequately, particularly given the technical challenges such interventions would have to overcome.

These possible arguments against somatic cell therapy do not respect adequately rights to freedom in scientific research, the duty to protect the vulnerable, and the rights to enjoy the benefits of scientific progress.

2.2 Ethical Oversight of Somatic Cell Therapy

The principal problems in somatic cell therapy involve adequate supervision both of the safety of research practices in the laboratory, and of the decision to initiate humans trials and the methods for securing full information from them.

We will not rehearse discussions elsewhere (e.g., Clothier Commission 1992) about the kinds of detailed knowledge and preparation that should be required before approving vectors and genes for clinical trials. From any ethical standpoint we know about, the central responsibility is to maximize the gains and minimize the risks, and ultimately decide whether or not the benefits realistically to be gained make legitimate asking others to run the risks involved.

Three points about this process are worth mentioning, because they flow from our belief that gene therapy will prove to be a broadly enabling technology. First, if we are right, new possibilities for its use will be hypothesized constantly. The desire to use the technology may not coexist with the knowledge of how to do it safely. The international community has a strong interest in facilitating transfer of appropriate assistance to maintain safety standards.

Second, periods of sudden enthusiasm for new medical technologies put pressure on the preservation of human rights in medical research. Physicians should not hastily adopt techniques without adequate preparation. Research protocols need interdisciplinary review with particular diligence, and a careful impartial weighing of risks and benefits. Experimental subjects need both full and fair explanation of the procedures, and honest information about the relative balance between research interest and therapeutic prospects. In particular, consent submissions that inform a patient "this may benefit you" - without disclosing realistically the many uncertainties, and the improbabilities of having solved all the technical barriers on the first try - should be avoided.

Third, discussions of consent to somatic cell therapy experiments in the literature seem driven, and driven to excess given the technology's present use, by models of experimenting on children with genetic disease. In such settings, of course, the requirements of prior proof of principle and realistic likelihood of success are maximal. A lifetime of treatment is contemplated in a person who cannot yet speak adequately for his or her own interests. But one should not fasten on adults who suffer genetic disease impossibly high standards for protocol initiation. As the influential Clothier Commission noted, we may foresee cases of terminally ill adults consenting to gene therapy research, recognizing its probable lack of direct therapeutic benefit. That model has long characterized cancer research (and perhaps too much so), but its prevalence may explain the relative ease of securing cancer protocol approvals.

The basic approach should be: the more that genetic therapy becomes like any other therapy, the more research review should be assimilated to conventional procedures when the techniques of gene insertion are standard ones. This process is underway in the United States, with the absorption of the Human Gene Therapy Review Committee into the parent Recombinant DNA Advisory Committee; the readiness by the RAC to permit "compassionate use" of gene therapy protocols, and the recent proposal and tentative agreement for FDA to take over from RAC, review of gene therapy proposals presenting no novel issues. The first

gene therapy protocol, Anderson's 1989 ADA experiment, was reviewed 15 times by 7 different regulatory bodies. A mere five years later many protocols can be approved on a standard FDA review.

2.3 Somatic Cell Therapies and the Problem of Enhancement Engineering

Recent gene therapy discussions insist that somatic cell procedures should be reserved for "serious disease". The Subcommittee concurs in that view because of the highly experimental nature of procedures, and the lack of sufficient experience for determination of the incidence and seriousness of side-effects that accompany various types of cellular alterations.

A cell performs complex chemical processes, through many pathways. It is far too soon for confidence that imposing on it a new "manufacturing" responsibility with energy requirements that must come from somewhere, will not somehow affect the way it (and through action on it, action on other cells) performs other tasks. Second, how stable is transfection? Do inserted genes always stay put or can they recombine and move to other cells, and if so how frequently does this happen and with what effects? It takes thousands of cases and years of experience for confident elimination of all the unhappy possibilities. No responsible ethics committee could at present approve a protocol directed at a disease it did not regard as serious.

What is a serious disease? Despite the diversity of cultures and world views, we believe the international community would reach virtual unanimity on a long list of serious diseases, and that list would include current targets of gene therapy protocols such as cystic fibrosis, ADA deficiency, Fanconi's anemia, cancer and AIDS. Moreover, some kinds of protocols that enhance human performance, for example by putting in genes that protect an individual against serious disease, must be conceptualized as protocols aimed at that serious disease, and not objected to as efforts to improve mere behavioral traits.

The differences of opinion about whether to classify disease as serious will be at the margin and at the borderline where disease prevention and lifestyle intersect. For example, is a protocol designed to reduce ordinary cholesterol levels aimed at a serious disease, if the serious disease could be avoided by eating less fat? Interestingly, the Report of the Norwegian Ministry of Health and Social Affairs to the Starting on Biotechnology Related to Human Beings (1992-93 at 69) accepts the limitation to serious diseases. The Report further notes that by implanting protective genes one may avoid disease, and suggests future "flexible limits", such as, for example, "implanting pigment cells in the skin to avoid sunburn".

Whether or not there is unanimity about what constitutes a serious disease does not seem to us an important issue in the international context. What matters is whether, within the particular society, the disease is defined as serious, so as to warrant the risks of experimental treatment.

The harder problem is that the "serious disease" limit is obviously used by commentators to avoid the necessity for present discussion of the ethics of using somatic cell gene therapy, at some future time, for purposes of enhancement. Does the serious disease limit rest on moral views about respect for human dignity, or is the prohibition simply that small gains do not warrant big risks? At some point, and measured in years rather than decades, the risk issues will be largely answered with respect to many of the technical aspects of gene therapy, particularly for uses that do not involve long-time maintenance with engineered cells. At that point, should somatic cell gene therapy procedures that are designed to enhance performance in some way or another be regarded categorically as violations of international human rights principles, or other moral values, and, if so, which ones and why?

One must start by noting that the medical model is a very expansive one. Many "conditions" become "diseases", or almost so, if, as and when, physicians develop the capacity to change them. Tooth decay is the "natural" fate of humankind, but we call it a disease and intervene to stop it. If a protein were found that overcame the "natural" loss of memory with age, (perhaps by strengthening CNS links that naturally fray with time, for illustration purposes only) then we would define the memory loss as a disease, and use the protein, and not conceptualize our activity as performance enhancement. Second, if some kinds of enhancement are wrong, the "wrongness" should not depend on the fact that "gene therapy" is the technology used to accomplish the result.

Somatic gene therapy must be analyzed by the general principles governing the uses and limits of medicine. The time will come when cells can be engineered for endogenous drug delivery, and then eliminated from the body by administering small molecule drugs, engineered to bind to the relevant cellular receptor-site that activates the inserted gene that causes cell death. Somatic cell "enhancement" therapies modeled on the use of such cells must be treated equivalently with whatever rules exist for drug use generally.

The "enhancement" case most frequently discussed in the bioethics literature is whether it is ethical to insert a gene to produce human growth hormone in a short but seemingly otherwise physically normal child. (We say seemingly because the bioethics literature constantly simplifies real world complexities to pose moral arguments more clearly. It is very hard to have assay systems that answer precisely what cells are doing. As just one example, the "normal" growth gene may be working, but some other gene, we know nothing about, may be limiting the protein's effect.)

The correct answer must be that if it is permissible to use the protein when the gene makes it in bacteria, it is permissible to use the gene when it is added to a cell, particularly because cellular administration of the gene may be safer. All gene therapy adds to enhancement as an ethical topic is the possibility that it may expand the range of enhancements possible, if, for example, some proteins require controls of dose, administration in sequence and specificity as to site of action, that could not be obtained by any other manner of administration.

Enhancements are problematic but ubiquitous in human society, from tattoos, to women and men piercing their ears to permit better display of their earrings. Guitarists may intentionally tear ligaments to permit the thumb to cover more frets. "Cosmetic" surgery has its uses and abuses. Social controls are imposed when "enhancements" may pose serious risks to health, and when they are thought unfair to others who are competing in a "game" which is defined so as to make their use an unfair advantage. The use of steroid drugs by athletes to build muscle mass is the common example. And, of course, for many or most societies, the use of public resources for such ends would be thought questionable in light of principles of just distribution.

There is a considerable literature on these topics that often gets pushed to the side as if somatic cell enhancement were a problem of a different sort. We think not. Particular uses of biochemical modulators must be examined against those models, if and when they are proposed.

3. Germ Cell Gene Therapy

Sperm and egg cells are different. They are the cells which go to form the individuals of the next generation and pass the genes from both parents to the offspring. They are the Germ Cells. Germ Cell Gene Therapy would involve the deliberate insertion of a gene into the germ cells, deletion of a gene from them, or alteration of a gene already there. Most people agree that germ line therapy raises serious ethical issues, because changes would be inherited.

3.1 Application to Germline Gene Intervention

Germline gene intervention (the term we use in preference to germline gene therapy, as "therapy" suggests the germline is somehow ill, which is not the case) has grabbed the world's attention. Imagining a world in which some people - the state, physicians, parents - have authority to select the genetic characteristics of the next generation, choosing chemical constituents to produce desired traits as if they were baking a cake, provides the conjurer with the occasion to reflect on the true nature of individual rights and human dignity. On the whole, thinking about ultimate values is a good thing. Yet such futures are often described in a way that ignores the moral values that inhere in the complexity and contingency of individual development. Mozart's genes do not guarantee Mozart's talent.

All major statements about germline intervention condemn its present use. That position is clearly correct. That genes can be put into animal germlines, and made to express, does not begin to answer safety issues for human use. Moreover, are there animal models that can predict the impact on the human brain? Enormous technical problems would have to be solved to make the technology realistic in light of the risks, particularly the control of gene expression throughout the organism's process of cellular differentiation. Moreover, one must have basis for predicting confidently the consequences of novel or altered genetic material in the workings of each and every cell type. So far as we know, there have been no efforts anywhere in the world to attempt germline intervention on human beings.

The prohibition on germline therapy is a matter of formal legislation in some nations (e.g., Sweden) and is accomplished through regulatory controls in many others, for example United Kingdom and the United States. Present prohibition does not deny the possibility of future use. Yet, there are important European documents that condemn germline therapy unequivocally. For example, Recommendation 1100 of the Council of Europe states: "Any form of therapy on the human germinal line shall be forbidden". Two important recent reports, the Clothier Commission (1992) and the Declaration of Inuyama (1990), however, do not categorically rule out germline interventions. In the United States, a number of prominent commentators believe that discussion should begin about developing interventions for the germline (Anderson and Fletcher; Wivel and Walters, 1993). Their call for present discussion reflects no disagreement about its present ethical impermissibility. It recognizes that the future policy process for approval will take a long time, and that more discussion now will make it more likely that appropriate policy will be in place in the future when technical capabilities are at hand. It may be right to debate the ultimate value questions. But those who suggest the desirability of germline therapy have not, the Rapporteurs believe, made a plausible case for any near term substantial use for it, and certainly no case that the gains would be worth the extraordinary efforts required.

Genetic intervention in the germline sounds most attractive as a technology for permitting a couple or individual to spare their descendants the burden of genetic disease. So, for example, a person at risk for a disease caused by a dominant gene might seek the procedure to guarantee that his future children would be free of it. If it is moral, as it surely would be, to remedy his condition by somatic cell gene therapy, why not cure it once and for all by germline techniques? Before considering the moral dilemmas inherent in that question, ask whether it is ethical to risk harm if there is a safer alternative. If one had the knowledge sufficient to attempt a germline intervention in these settings, it seems certain one would know how to accomplish the same end without employing germline techniques.

Treating the adult's cells *in vivo* to remove the gene is a technology not even envisaged. By contrast, sorting sperm (or eggs) using DNA probes to separate those that carry the gene from those that do not falls into the category of not presently feasible. If it could be done, however, one could solve the problem by using "good" sperm and discarding rather than repairing sperm that carried the gene. (Similarly, research - published after this paper was prepared and discussed by the IBC - suggests the possibility of identifying sperm stem cells, and perhaps altering them ex vivo, and restoring those grown from cells that were successfully repaired.)

The probable starting point for germline intervention, however, is the human zygote at the four cell stage, fertilized through *in vitro* fertilization. Certainly, that is the only present way to do it. Animal models suggest that gene insertion that will differentiate into every cell is feasible. But if a particular zygote can be identified as carrying (or lacking) the gene, and therefore be appropriate for treatment, why would one try the extraordinary procedure of repair rather than selecting for implantation a zygote that did not have the gene? Such screening procedures are in use, on an experimental basis, in a few hospitals around the world. For example, at a hospital in Israel, procedures for selective screening of zygotes,

using so-called PCR technique to test the DNA in one cell of the four-cells was used to screen for cystic fibrosis.

There are many who strongly oppose any selection or discarding of zygotes, and for these persons that opposition is itself a sufficient basis to protest the development of germline interventions. Opposition to selecting zygotes goes hand-in-hand with opposition to non-therapeutic experiments on zygotes that will never be implanted. For example, German law prohibits non-therapeutic experiments on the human zygote. While all acknowledge the special respect owed to the fertilized egg throughout its development, what actions are required or prohibited by that respect is an issue on which the world community is deeply divided. This Report does not attempt argument on the issue. Obviously, it is crucial for germline interventions. No human germline interventions can be safely developed, we think, without the opportunity to test whether genes inserted somewhere (sperm, egg, zygote) in fact are replicated appropriately in the first stages of cellular differentiation.

The largest plausible need for germline intervention is to treat affected zygotes, once and for all, on behalf of persons who disapprove of abortion or selective implantation. If safe interventions were developed over their protest, these persons might wish to have the procedure done in preference to selective abortion or implantation, which they believe is immoral. We would think, however, that developing a technology to serve principally those who disapprove its development on the deepest moral grounds, is a public policy option with limited appeal. There may be other needs. There may be couples where each partner is homozygous for a particular recessive gene, say the cystic fibrosis gene. For this couple, no "normal" children are possible because no copy of the gene is present in the DNA of either. There may be such couples, but the prospect of launching a technological enterprise so vast to serve them seems too implausible to justify relaxation of the prohibition on developing germline intervention. There may perhaps be other conditions where the alternative is not feasible, but again, so far as we are aware, they are very rare. If we correctly understand the limited plausible uses, the need to initiate policy revision now for later seems lacking.

That said the uncertainties both of scientific knowledge and the pace of technical improvement cautions against saying "never" unless there is some important moral value to be served. For example, if it were discovered that some new virus entered brain cells early, and produced dementia after a latency period, is it inconceivable that germline intervention might be the best (and only) way of protecting humans from it? New viruses are obviously possible. Who knew that retroviruses even existed forty years ago? Therefore, although we are not convinced that the policy process needs to move forward now, it seems appropriate to consider whether there are sound reasons for categorically barring germline interventions. Do concepts of human dignity and respect for human life require categorical condemnation of germline intervention?

Protection of human dignity and worth puts important limits on tampering with the reservoir of potentialities inherent in the gene pool. But are categorical prohibitions desirable? They might be based on:

- the need to experiment on zygotes. We will not comment further on this issue;

- impermissible risks to the future child.

There is much merit in this view. Any experiments will necessarily require inferences from animal models, but what kind of animal model can adequately indicate the effects of altering every single cell in the body, and what effect those alterations may have on uniquely human qualities such as cognition? One might say, categorically, that the risks will always be too great to justify intervention, and certainly so against conditions that have long been thought part of the human condition, such as memory loss with age. After all, there is no present patient who is sick and in need of treatment, whose benefit is to be weighed against risk.

The philosophical question of whether or not future persons have a "right" not to be experimented with before they are persons with interests of their own is a deeply interesting philosophical one. So too whether their hypothesized interest in disease free existence can be weighed in the balance against the risks. Whether or not the language of "rights" is appropriate, however, common language regards people as "wronged" when they are injured by activities that occurred before their conception, regardless of whether or not they have the right to maintain a lawsuit. We think, for example, of preconception exposure to toxic substances. If one can think of the interests of future persons this way, then the presumptive legitimacy of parental consent to speak on their behalf is consistent with the rest of our institutional arrangements.

The issue then is whether there are not disabilities so severe that one may impute to parents the right to speak for their descendants, and legitimate risk-taking on their behalf. We think there are. Moreover, we think that to invoke a child's right to an unaltered genetic makeup so as to visit on him conditions incompatible with prolonged life is too paradoxical to be accepted.

The third objection to germline interventions is that mistakes cannot be recalled. If, for example, in tampering with the organism's genes, scientists somehow launch a new genetic problem on the world, the gene they create may spread its way throughout the population. In effect, adding a new gene to this zygote is research to which I, a third party, have not consented on behalf of my future children, who may be affected by an accident. Such theoretical logic as this position may have, however, ignores the flux of the human gene pool, treating it as a work of art rather than an on-going process. We do not think the person who is exposed to radiation is running risks for which he needs our consent, on the theory that he might be burdening our common descendants. What makes germline intervention problematic is the intentional character of the enterprise.

A final argument against germline intervention is that it is somehow beyond human right to interfere with the fundamental process of life, and that this prohibition cannot be overcome by the highly ambiguous notion of "diseased" genes. To be creditable, this argument must say why intentional interference with the germline poses moral issues sharply different from the unplanned, but nonetheless significant impact of human activities on it. It must also insist on such a distinction in the face of the child's suffering ills that by hypothesis might have been safely prevented.

3.2 Germline Enhancement

The prospect of using germline interventions to improve the basic traits of humanity is everywhere condemned. We believe it impermissible also, for standard reasons. First, we cannot hypothesize how issues of risk versus benefit could possibly be weighed to favor

proceeding. The plausible uses of germline intervention, even coupled with extensive experience with somatic cell therapy, would not ground experience that this kind of intervention can be safely done. There can be no "suicide genes" here. Therefore, although we can imagine some somatic cell enhancements that might not be wrong, it would be just as wrong to force them on our children's children as it would be to forcibly medicate an adult with a drug. Secondly, human individuality and community is linked to the claim that you are who you are, the product like all of us of chance. It is wrong to burden individuals with a programmed destiny, and make them victims of genetic expectation. Finally, for one set of humans to claim to foresee what traits the world will need in the distant future is wrong.

4. Administering gene therapy

Successful gene therapy requires that the:
• genetic malfunction/nature of a disease is understood;
• therapeutic material can be delivered to the target cells in the affected tissue or organ;
• therapeutic material is active for the intended duration and delivers the intended benefit to the target cells;
• harmful side effects, if any, are manageable.

Therapeutic material can be delivered to the target cells in two main ways. First, it can be inserted into cells from the affected tissue outside the body, and these cells then returned to the body. Second, it can be delivered directly into the body at the required site. Either way a 'delivery vehicle' called a vector is used to get the therapeutic material to the patient's target cells. Vectors are most commonly based on modified viruses, because these can target and enter cells efficiently. To date, some 70% of gene therapy trials approved have involved viral vectors.

5. Clinical trials

Ninety-six trials involving patients have been approved in the UK since 1993. Of these, 72% were for cancer, 13% for single-gene disorders and 7% for vascular disease. The majority were 'Phase I' clinical trials to evaluate the safety of a proposed gene therapy with small numbers of very ill patients, for whom no other treatment options existed. For instance, some early CF trials had 16-18 patients. If Phase I is successful, a trial may be repeated with a larger group of patients in Phase II, to assess therapeutic activity in very ill patients. Even at this stage, a successful outcome cannot be attributed solely to the efficacy of the gene therapy. Efficacy can only be established in a Phase III clinical trial, involving more (perhaps up to a few hundred) patients. To date, three UK gene therapy trials – one for HIV and two for glioma (a type of brain cancer) – have progressed to Phase III.

6. Marketing approval

No form of gene therapy has yet advanced to become a licensed treatment in the UK. Any commercial marketing of a gene therapy product would require a marketing licence from the European Medicines Agency (EMEA). As a human medicinal product of biological origin, gene therapy medicinal products for marketing in one or more of the EU member states must receive approval via the EMEA's centralised marketing procedure. This is a well established procedure for biological therapeutic products, such as recombinant therapeutic proteins. To assess each application for marketing authorisation, the EMEA appoints a

rapporteur and a co-rapporteur from two member states. The application is then assessed on the basis of quality, safety and efficacy of the product.

7. Issues

Gene therapy is only used to treat very serious diseases. While the first clinical trials focused mainly on single gene disorders, more recent trials have looked at complex diseases such as cancer. Such trials offer hope for cancer patients but are at an early stage. The experience of 12 years and nearly a hundred UK trials in gene therapy has produced significant successes. But it has also highlighted questions about effectiveness, safety, durability and the likely commercialisation of gene therapy. These are discussed below.

8. Technical considerations
8.1 Gene delivery

Successful gene delivery is not easy or predictable, even for single-gene disorders. For example, although the genetic basis of CF is well known, the presence of mucus in lungs makes it physically difficult to deliver genes to the target lung cells. Delivering genes for cancer therapy may also be complicated where the disease may be in several sites. Gene therapy trials for X-chromosomelinked severe combined immuno-deficiency (X-SCID) however, have been more successful. In this case, the genetic basis of the disease is well-understood and the therapeutic material can be delivered using the established procedure of bone marrow transplant. A viral vector has been used to introduce functioning copies of the gene whose malfunction causes X-SCID into blood producing stem cells from the patient's bone marrow. These are then transplanted back into the patient. In most cases the modified bone marrow successfully supplied the missing gene product, and the patients are able to lead normal lives.

8.2 Durability and integration

Some gene therapy approaches aim to achieve a long term effect. Where such durability is required the therapeutic material must remain functional for the intended duration or gene therapy may not achieve long term benefits. Two possible ways of achieving this are to use multiple rounds of gene therapy, or to integrate therapeutic genes so they remain active for some time. Integrating therapeutic DNA into the target cells' genetic material, while long-lasting, raises concerns over possible undesirable side effects. For instance, this approach has been used in trials to treat babies with X-SCID syndrome.

In UK trials, 7 babies responded well to gene therapy and remain healthy. However, 3 of those in a similar French trial went on to develop leukaemia-like symptoms. A possible explanation is that the therapeutic material might have integrated where it could affect another gene to produce rapid growth of cancerous cells. Researchers are thus investigating ways of achieving long-term therapeutic effects without integration, for instance by using stable, non-integrating, vectors.

Other approaches seek more immediate effects, where integration of therapeutic DNA into the target cells is not the aim. For instance, in gene therapy to treat cancer, the aim may be to use 'suicide' genes to kill cancerous cells as quickly as possible.

8.3 Immune response

When a viral vector is used to deliver gene therapy, the body may recognise it as 'foreign' and mobilise the immune system to attack it. In cancer, triggering such an immune response may be the aim of the gene therapy. In other cases, immune responses may reduce the efficacy of gene therapy, causing the patient to stop responding after a few applications or inducing serious side-effects. Further, an enhanced response to vectors encountered previously may make it difficult to give repeat applications of gene therapy.

8.4 Safety of vectors

Worldwide, more than 3,000 patients with serious diseases have been treated using experimental gene therapies since 1990. To date, there have been just two fatalities that have been directly attributable to such treatments. Nevertheless, a small minority of cases have raised potential concerns. For instance, the use of viral vectors has been suggested as a factor in the death of a US gene therapy patient and the cases of leukaemia-like symptoms after X-SCID gene therapy trials in France.

Animal research may also provide important information on vector safety. Following recent reports of a preclinical study, where mice developed liver tumours after exposure to one particular vector, GTAC published an open letter drawing attention to potential safety concerns. Further the UK Health and Safety Executive issued information and interim advice on containment. These steps were taken in case these observations affected any pre-clinical or clinical studies planned or in progress outside the UK; they do not appear to affect any current or previous UK trials.

8.5 Uncertainty

While GTAC and MHRA make every effort to maximise patient safety, uncertainties remain. The range of explanations that have been advanced to account for leukaemia-like symptoms in the French X-SCID gene therapy trial, illustrate the difficulties faced by regulatory bodies. These explanations include problems with the integration of the therapeutic material, an immune response to the vector, a family history of cancer, and pre-existing infections and other problems symptomatic of immune deficiency.

9. Regulatory challenges
9.1 Gene therapy research on children

Conducting medical research with children raises difficult issues. To date, very few gene therapy trials have involved babies or children, but this may change. There may be good clinical reasons to involve children in gene therapy trials. For instance, some diseases progressively worsen with age, and the best chances of positive outcomes often lie with interventions at an early stage. CF is a case in point. It is irreversible after a certain stage of deterioration of the lungs, so it is only possible to stabilise (rather than improve) a 30 year old patient's condition using conventional treatments.

Gene therapy for CF may thus work best in the early years of life. In such cases, researchers are required to demonstrate the therapeutic necessity of using children in a gene therapy trial.

Obtaining informed consent for a child to take part in a clinical trial is legally complex and there is ongoing debate in the UK on this issue. GTAC's guidelines on writing patient

information leaflets include guidance on how to communicate information to the child. The Medical Research Council (MRC) has also published an ethics guide for medical research involving children.

9.2 Accountability, openness and transparency

GTAC and MHRA adopt a case-by-case approach to safety issues. For instance, following reports of leukaemia-like illness in three patients in a French X-SCID trial, GTAC and the MHRA's Committee on Safety of Medicines made joint recommendations to the Department of Health (DH). While such an approach is welcomed by researchers, groups such as GeneWatch have been critical of a lack of transparency in the UK's gene therapy regulation. For instance, it points out that GTAC holds its meetings for evaluation of research proposals in private. Furthermore, MHRA publishes details of clinical trial authorisation applications in a database accessible only to competent European regulatory bodies, the EMEA and the Commission. GTAC argues that it publishes minutes of meetings and an annual report, but has to preserve independence in evaluation, protect patient privacy and, in later phases of trials, ensure commercial confidentiality. MHRA believes that confidentiality encourages the reporting of sensitive information relating to public health concerns. All NHS funded trials must be registered in a publicly accessible database. Industry has also announced voluntary measures on registering Phase III and further clinical trials.

9.3 Resources

96 gene therapy trial applications have been approved in the UK since 1993. However, as investment in research grows, the number of trial applications could also grow in the next few years. This could have resource implications both for GTAC and MHRA.

9.4 Future regulatory challenges

As the science of gene-based medicines evolves, new technologies and new therapies will emerge. For instance, RNAi is a method of 'silencing' genes to prevent the formation of unwanted protein. MRC is investing in basic research into the therapeutic potential of RNAi. At present, GTAC and MHRA review the use of emergent technologies in clinical trials on a case-by-case basis. New interventions combining gene therapy with other approaches such as stem cell therapy are emerging. For instance, bone marrow stem cells are already used in some gene therapy trials. While bone marrow transplants are an established practice, it is possible that in the longer-term, gene therapy trials could involve stem cells derived from embryos. Such developments may pose new challenges to the regulatory system, which currently regulates gene therapy and embryonic stem cell research separately. The House of Lords Stem Cell Committee1 considered ways of regulating new stem cell therapies and identified a number of options. These included expanding the remit of GTAC to take on this role, or establishing a new regulatory body based on the GTAC model. DH has noted that it will consider the need for further oversight in this area in the light of any new developments.

10 Future prospects and expectations
10.1 Research

In its 2003 White paper, the Government committed £10 million for research into single gene disorders and safety of vectors, as well as to enable access for public sector researchers to high standard gene therapy vector production facilities2. In 2004, the

Government awarded £0.5 million for haemophilia, £1.6 million for Duchenne Muscular Dystrophy, £0.9 million for childhood blindness and £1 million for research into long-term safety of some techniques used for gene therapy. In addition, MRC funds basic science such as vector development and delivery methods in cell lines and animal models. Public funding of gene therapy research is supplemented by funding from research foundations and charities. Over the last five years, the Wellcome Trust has provided £4.8 million for non-clinical gene therapy research aimed at several inherited and acquired diseases. Cancer Research UK (CR-UK) is also currently funding nine gene therapy research projects. While patient groups are largely satisfied with the current disease-led approach to gene therapy research, researchers have called for more research into vector safety, delivery techniques, molecular causes of diseases, and uncertainty of outcomes.

10.2 What happens next?

Opinions vary as to how soon commercial gene therapy products will be on the market. For instance, CR-UK believes that gene therapy presents realistic opportunities for new ways to treat cancer, but not for the next 10-15 years. The Genetic Interest Group, a national alliance of patient organisations, does not expect that working therapies for a wide-range of diseases will emerge in the near-term, but suggests that research will contribute to the knowledge of vectors, insertion and delivery techniques, and safety. Such understanding will be critical to the development of potential gene therapies.

However a number of gene therapy products may be in the pipeline. For instance, a UK firm, Ark Therapeutics, has two gene-based medicines in late stage clinical trials; it currently aims to apply to market these in 2007. The first ever recombinant gene therapy to obtain marketing approval was 'Gendicine', a gene-based medicine for treating head and neck cancer. As noted earlier, any gene therapy products will have to be assessed through the EMEA's centralised procedure. While well-established for other biological therapeutic products, this procedure has not yet been used for gene therapy products. If the UK regulator (the MHRA) is not appointed as the Rapporteur or Co-rapporteur, it will still review the data provided by the applicant company and provides inputs into the process of authorisation through the Committee for Human Medicinal Products (CHMP).

10.3 Ethical considerations

While the body has many billions of cells, only a very small proportion of these cells are involved in reproduction, the process by which our genes are handed on to future generations. In males these cells are located in the testes and in females, in the ovaries. These special reproductive cells are called 'germ cells'. All other cells in the body, irrespective of whether they are brain, lung, skin or bone cells, are known as 'somatic cells'.

In gene therapy, only somatic cells are targeted for treatment. Therefore any changes to the genes of an individual by gene therapy will only impact on the cells of their body and cannot be passed on to their children. Changes to the somatic cells cannot be passed on to future generations (inherited). Somatic gene therapy treats the individual and has no impact on future generations.

The IBC has many potential tasks, from providing information to suggesting principles for international legal recognition. In its ethics evaluative role, it must suggest ethical

principles that are grounded in universal ideas even as it takes account of the happy diversity of cultural and religious traditions that co-exist in the world.

The principles and materials of international human rights law may yield a rich supply of doctrines for evaluating what should be done in discrete bioethics cases. An interesting recent Report, "The Promotion of Human Rights in the Life and Health Sciences, Recommendations to the United Nations", organized by Audrey Chapman, contains suggestive discussions of this point. Human rights law contains provisions that are analogous to the principles that flow from analysis of moral obligations implicit in doctor-patient relationships, which is the starting point, for example, of much of the Anglo-American bioethics literature, as well as the bioethics traditions in other communities. Moreover, and happily these various traditions converge in their treatment of experimental medicine, our principal field where there is substantial consensus on the questions to ask, and the procedures to follow.

In appraising gene therapy, these principles, at minimum, must be taken account of, and built upon. Each is drawn from international instruments:

1. the respect for human dignity and worth;
2. the right to equality before the law;
3. the protection of rights of vulnerable individuals;
4. the right not to be subjected without free consent to medical or scientific experimentation;
5. the right to the highest attainable standard of physical and mental health and associated rights to health care;
6. the right to protection against arbitrary interference with privacy or with the family;
7. the right to enjoy the benefits of scientific progress and its application; and,
8. the right to freedom for scientific research.

In as much as all present gene therapy constitutes medical and scientific experimentation, (and a rather extreme form of it), the right "not to be subjected without free consent" to it is guaranteed. "Free" consent implies informed consent, with no coercion. The duties imposed on researchers and procedures for implementing them have been spelled out in other internationally significant documents. The Nuremberg Code was the foundation. That Code was formulated in the unusual context of the international war crimes trial, for purposes of stating the internationally-recognized principles that might permit researchers to engage in conduct that would otherwise be a violation of subjects' rights (and indeed, where injury was risked or caused, a serious crime). The Declaration of Helsinki, prepared by the World Medical Association, derived from and builds on the Nuremberg Code. In turn, the World Health Organization (WHO) and the Council for International Organizations of Medical Science (CIOMS) based their influential "International Guidelines for Biomedical Research Involving Human Subjects" on the Helsinki Declaration. The guidelines' purpose is to indicate how fundamental ethical principles should guide the conduct of biomedical research involving human subjects. In its most recent version (CIOMS/WHO, 1993), one finds as "general ethical principles", the proposition that: "all research involving human

subjects should be conducted in accordance with three basic ethical principles, namely respect for persons, beneficence and justice".

This is precisely the language of the Anglo-American tradition with respect to human experiments, stressing principles of autonomy, cost-benefit analysis, and justice in distribution of benefit and risk.

11 Overview

- Gene therapy can potentially treat diseases such as CF, cancers, heart disease and HIV infection. To date, no gene therapy clinical trial has given rise to the development of a commercial treatment in the UK.
- While industry considers commercial gene therapy likely for some cancers in the next few years, others suggest it may be 10 to 15 years before gene therapies are widely available.
- Potential issues with gene therapy include effective delivery, longevity of the therapy and safety concerns.
- Gene therapy clinical trials in the UK are regulated by GTAC and MHRA. MHRA is also responsible for regulating medicinal gene products in the UK.
- The regulatory system considers all gene therapy research proposals on a case-by case basis, taking ethical and scientific factors into account. Some have called for more open and transparent assessments.
- Technical developments may necessitate changes to the current regulatory framework, which has separate arrangements for therapies derived from human genes, stem cells, cells and tissues.

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Chapter 16

Gene knockout

1. Introduction

A gene knockout (KO) is a genetic technique in which an organism is engineered to carry genes that have been made inoperative (have been "knocked out" of the organism). Also known as knockout organisms or simply knockouts, they are used in learning about a gene that has been sequenced, but which has an unknown or incompletely known function. Researchers draw inferences from the difference between the knockout organism and normal individuals. The term also refers to the process of creating such an organism, as in "knocking out" a gene. The technique is essentially the opposite of a gene knock-in. Knockout is often abbreviated as KO. Knocking out two genes simultaneously in an organism is known as a double knockout (DKO). Similarly the terms triple knockout (TKO) and quadruple knockouts (QKO) are used to describe 3 or 4 knocked out genes, respectively.

2. Knock-out Methodology

Knockout is accomplished through a combination of techniques, beginning in the test tube with a plasmid, a bacterial artificial chromosome or other DNA construct, and proceeding to cell culture. Individual cells are genetically transformed with the DNA construct. Often the goal is to create a transgenic animal that has the altered gene. If so, embryonic stem cells are genetically transformed and inserted into early embryos. Resulting animals with the genetic change in their germline cells can then often pass the gene knockout to future generations.

The construct is engineered to recombine with the target gene, which is accomplished by incorporating sequences from the gene itself into the construct. Recombination then occurs in the region of that sequence within the gene, resulting in the insertion of a foreign sequence to disrupt the gene. With its sequence interrupted, the altered gene in most cases will be translated into a nonfunctional protein, if it is translated at all.

A conditional knockout allows gene deletion in a tissue or time specific manner. This is done by introducing short sequences called loxP sites around the gene. These sequences will be introduced into the germ-line via the same mechanism as a knock-in. This germ-line can then be crossed to another germline containing Cre-recombinase which is a bacterial enzyme that can recognize these sequences, recombines them and deletes the gene flanked by these sites.

Because the desired type of DNA recombination is a rare event in the case of most cells and most constructs, the foreign sequence chosen for insertion usually includes a reporter. This enables easy selection of cells or individuals in which knockout was successful. Sometimes the DNA construct inserts into a chromosome without the desired homologous recombination with the target gene. To avoid isolation of such cells, the DNA construct often contains a second region of DNA that allows such cells to be identified and discarded.

In diploid organisms, which contain two alleles for most genes, and may as well contain several related genes that collaborate in the same role, additional rounds of transformation and selection are performed until every targeted gene is knocked out. Selective breeding may be required to produce homozygous knockout animals. The effect of

the absence of a gene can be very informative about the normal function of the gene. Eliminating a gene (gene knock-out) completely from a diploid organism requires knocking out both copies of the gene in the cells. There are a variety of methods for producing gene knockouts in different model organisms.

Knock-in is similar to knock-out, but instead it replaces a gene with another instead of deleting it.

3. Knockout animals

"The purposeful deletion of a specific gene for studying its functions is knocking out Very useful in understanding role of a specific gene in a disease".

3.1 Knockout Mouse

A mouse in which a very specific endogenous gene has been altered in such a way that interferes with normal expression, i.e., it has been knocked out.

Knockout Mice are produced to:

- To study effects of gene products, biochemical pathways, alternative compensatory) pathways and developmental pathways
- To recreate human diseases in animals to establish models to test the beneficial effects of drugs or gene therapy.

3.2 Procedure for Generating a KO Mouse

Gene alteration in KO mice is targeted to very specific genes. DNA must integrate at precise positions in the genome. Integration of the altered gene takes place in embryonic stem cells ex vivo. Verification of exact location of integration occurs before the ESC is introduced into blastocysts to become part of the developing embryo.

- DNA that has been engineered to contain a mutant copy of the gene is introduced into special embryonic stem cells (ES cells) that were growing in tissue culture.

- Cells that take up the DNA are tested to find those in which the mutant copy has replaced one good copy of the gene.

- Cells with one mutant copy are introduced into an early embryo (blastocyst) that will take up these cells.

- Mice that are born from this manipulation (and contain the one mutant copy in their germ cells) are mated to each other.

- One in four mice from this mating will contain two mutant copies of the gene (Figure 1).
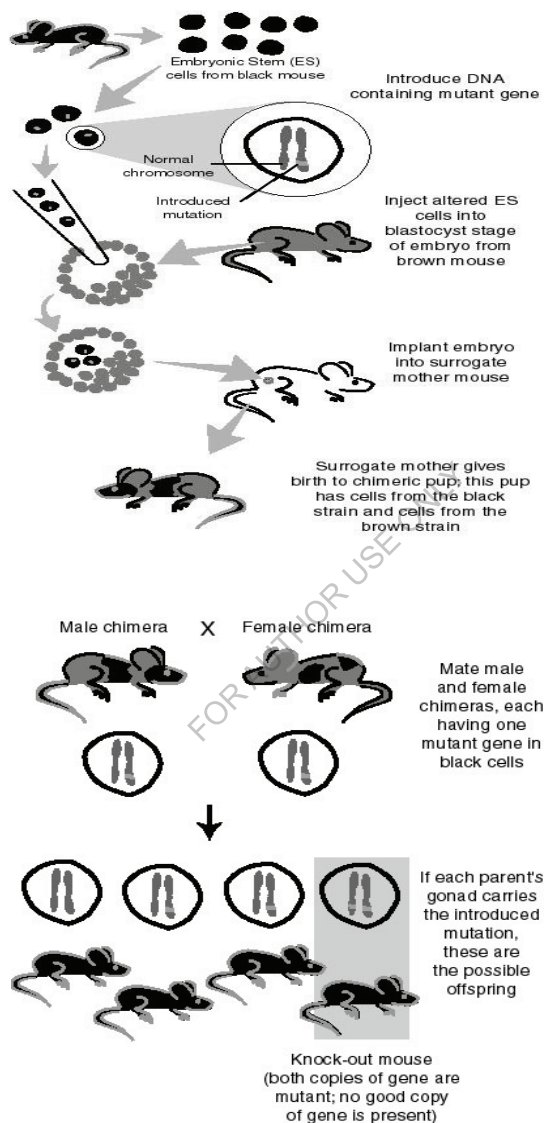
Figure 1 Engineering a Knock-out Mouse

3.3 Mice knocked out for

a. Recombination Activating Gene 1 Knock Out
Development

A neomycin resistance cassette was inserted in the RAG-1 gene, resulting in a 1356 bp deletion in the 5' end of the coding sequence into 129-derived AB1 ES cell line. The C57BL/6J strain was generated by backcrossing mice carrying the Rag-10 mutation 10 times to C57BL/6J inbred mice. Rag-1 deficient mice were transferred to the NOD/LtSz strain background.

Description

RAG-1deficient mice are viable and fertile. They are unable to initiate V (D) J recombination in Ig and TCR genes and lack functional T and B lymphocytes. Although RAG-1 expression has been reported in the central nervous system of the mouse, no obvious neuroanatomical or behavioral abnormalities have been found in the RAG-1- deficient mice. On the NOD background, NOD.Rag10 mice are devoid of mature T or B cells. NOD/LtSz-Rag10 recipients of adoptively transferred spleen cells from diabetic NOD/Lt+/+ mice rapidly develop diabetes.

b. T-cell receptor alpha chain Knock Out
Development The Tcrao targeted mutation was generated in GK129 ES cells (derived from substrain129P2/OlaHsd) via homologous recombination. The neomycin resistance gene was inserted into the first constant region of the Tcra gene. The Tcratm1Mjo allele on the C; 129 mixed background was then transferred to the NOD background. The line is currently at the 12th backcross to NOD/LtJ.

Description

The mutation prevents expression of any endogenous TCR alpha chains and thereby blocks differentiation of alpha beta T cells. NOD mice homozygous for the Tcra targeted mutation lack alpha beta T cells and are completely protected from diabetes development. Because of the complete elimination of alpha beta T cells, these mice are useful in adoptive transfer experiments or in crosses to TCRab transgenic lines.

c. Immunoglobulin IgM Knock out
Development

The membrane exons of the gene encoding the mu-chain constant region were disrupted by insertion of a neomycin resistant gene. The mutation was then transferred from the original chimeric stock with a mixed 129/Sv and C57 BL/6 genome onto the NOD/Lt background.

Description

Heterozygous mice are normal and fertile. In homozygous animals however, B-cell development is stopped at the stage of pre-B-cell maturation and B cells are therefore absent. No IgM is present on the pre B cell surface. NOD.μMTdeficient mice have normal numbers of T cells but are free of overt Diabetes and insulitis resistant. The frequency of disease in the B lymphocyte intact segregants is equivalent to that of standard NOD mice. Homozygous μMT knock out animals display a high incidence of lymphoma of both T- and B-cell origin compared with these mutations on other genetic backgrounds. The lymphoma incidence in both strains is greater in females, reflecting the greater incidence of autoimmune type 1 diabetes in NOD females than in males.

d. Interleukin 4 Knock Out
Development

A neomycin resistance cassette was used to disrupt the first exon of IL4 into E14-1 embryonic stem cells. The mutation was maintained on a B6;129 segregating background until it was transferred to NOD/Lt using a marker assisted protocol.

Description

Mice homozygous for the IL4 targeted mutation are viable and fertile. T and B cell development is normal but IgG1 and IgE levels and the ability of homozygous mutant mice to produce Th2-derived cytokines are significantly reduced. On the NOD background, the IL-4-null mutation does not accelerate or intensify insulitis in regular NOD and has no effect on the timing or frequency of the transition to diabetes. Coxsackievirus B4 (CVB4) infection in NOD mice leads to acceleration of Type 1 diabetes. When NOD.IL4-/- mice are infected with CVB4at 12 weeks of age, the onset of diabetes is accelerated in

NOD.IL4-/- mice compared to NOD. IL4+/+ mice. In the absence of an insulitic threshold, CVB4 infection leads to long-term disease protection. This protective mechanism is not affected by the disruption of IL4 gene.

e. Interleukin 10 Knock Out

Development

The first exon of IL10 was disrupted after the fourth codon, a neomycin resistance cassette, and an additional termination codon in the third exon, was used for homologous recombination in E14-1 ES cells (129P2/OlaHsd-derived). The mutation was then transferred to the NOD/Lt background using a marker assisted protocol. NOD/Lt mice heterozygous for this IL100 allele and homozygous for diabetes susceptibility loci (Idd) were intercrossed to develop mice homozygous for IL10tm1Cgn and all Idd loci.

Description

Mice homozygous for the IL10 targeted mutation are viable and fertile when housed under SPF conditions. This mutant develops type 1 diabetes at the same rate as the NOD/Lt parental strain. The Il10 mutation also renders this line susceptible to colitis (although not as severe as other strains of Il10 deficient mice when maintained under standard housing conditions.

f. Gamma Interferon Knock Out

Development

The targeting vector has a neomycin resistance gene inserted into exon 2, which introduces a terminaison codon after the first 30 amino acids of the mature IFN-γ protein. The targeting vector was transferred into AB-1 embryonic stems. The mutation was then transferred to the NOD background.

Description

Deficient mice develop normally and are healthy in the absence of pathogens. However, mice deficient for IFN-γ have impaired production of macrophage antimicrobial products and reduced expression of macrophage major histo-compatibility complex class II antigens. NOD Mice homozygous for the IFN-γ KO targeted mutation are viable and fertile. The genetic absence of IFN-γ does not prevent either insulitis or diabetes in the NOD mice, but increases the time to onset. Splenocytes taken from IFN-γ deficient diabetic mice are fully capable of transferring diabetes to naive recipients. In both NOD. IFN-γ -/- and NOD. IFN-γ - /+ mice, IL-12 administration generates a massive and destructive insulitisand increases the number of pancreatic CD4(+) cells. NOD IFN-γ 0 homozygous mice do not display increased acinar cell apoptosis and abnormal salivary protein expression, typically observed in parental NOD mice before Sjogren's syndrome-like autoimmune exocrinopathy. When NOD. IFN-γ -/- mice is infected with Coxsackievirus B4, Insulitis or diabetes development is delayed by several

weeks compared to NOD mice. When mice are infected at 12 weeks of age, neither acceleration nor long-term protection is elicited in NOD. IFN-γ - /- mice.

g. Gamma Interferon Receptor Knock Out
Development
The IFN-γR gene was disrupted by inserting the neomycin resistance gene into exon 5, which encodes an extra-cellular membrane proximal portion of the Receptor. Mice carrying the IFN-γ null mutation were backcrossed with NOD mice with intercrosses to produce experimental animals (Wang B *et al*, Mathis D, 1997). The line is at the >9th backcross in NOD/LtJ.

Description
Mice without the IFN-γR have no overt anomalies, and their immune system appear to develop normally. However, mutant mice have a defective immune resistance, despite normal cytotoxic and T helper cell responses. NOD mice homozygous for the IFN-γR0 mutation show a marked inhibition of insulitis, both in the kinetics and penetrance, and no signs of diabetes, either spontaneously or after experimental provocation. However, extended backcrossing of this mouse line to the NOD mouse result in a segregation of the IFN-γ R-deficient genotype from the diabetes-resistant phenotype. These results indicate that the protection of NOD mice from the development of diabetes is not directly linked to the defective IFN-γR gene but, rather, is influenced by the presence of a diabetes-resistant gene(s) closely linked to the IFN-γR loci derived from the 129 mouse strain. Outcross studies of NOD mice with several different non autoimmune prone strains, including B6, have identified multiple polymorphic genetic loci conferring susceptibility or resistance to Type 1 diabetes.

Chapter 17
Animals for Human Health Purposes

1 Introduction

As genetic engineering has the potential to alter the uses to which domestic animals are put, it also can lead to fundamental changes in the relationship between (1) individuals of the same species or population, (2) different species, (3) engineered animals and their products, and (4) the products and humans. There currently are major research efforts underway to develop the use of genetically engineered animals as sources for production of non-traditional materials for human use. Such uses can be divided into three major categories: biopharmaceuticals for animal or human use; live cells, tissues, and organs for xenotransplantation; and raw materials for processing into other useful end products. Several possible concerns that might in practice arise from the first two uses are discussed in the following sections. This can be called as "gene pharming" which means the use of transgenic animals (and sometimes plants) to produce medicine.

2 Biopharmaceutical Production

A large number of genes encoding useful protein products-hormones, blood proteins, and others has been introduced into domestic animals, leading to their expression in milk, eggs, or blood (Table 1). So far, none of these animals has been used for commercial production. However, a recent report suggests that the same technology might be extended to the large-scale have the potential to use well-established agricultural methods to produce large amounts of valuable products at relatively low expense as compared to fermentation. Although the end products of these applications will be novel, by and large, the process of production and the potential concerns are not likely to differ greatly from those seen in current practice, such as the use of animals or animal cell cultures to prepare live vaccines, hormones, or traditional products such as meat, milk, or leather. These standard products are subject to specific regulatory procedures, and essentially the same regulatory framework is expected to apply for products of both biopharming and standard technology as regards common issues such as purity of the final product, microbial contamination, levels of adventitious DNA, and the like. Nevertheless, a few more specialized concerns arise.

Table 1: Potential uses of transgenic animals for pharmaceutical production

Species	Theoretical Yield (g/yr of Raw Protein)	Examples of Products Under Development
Chicken	250	Monoclonal antibodies
		Lysozyme
		Growth hormone
		Insulin
		Human serum albumin
Rabbit	20	Calcitonin
		Superoxide dismutase
		Erythropoietin
		Growth hormone
		IL-2
		á-glucosidase

		Antithrombin III
		Tissue plasminogen activator
Goat	4,000	Monoclonal antibodies
		á-1-Antitrypsin
		Growth hormone
		á-1-Antitrypsin
Sheep	2,500	Factor VIII
		Factor IX
		Fibrinogen
		Human serum albumin
Cow	80,000	Lactoferrin
		á-Lactalbumin

3 Contamination or Spread of Novel Pathogens

There is a theoretical potential for microorganisms to acquire-by recombination or transduction-genes from the vector constructs used to insert the transgene. Although there is no example yet of acquisition of any gene, including drug resistance markers, by bacterial flora living in a transgenic animal, the spread of introduced genes remains a possibility, albeit remote.

Of greater concern is the possibility for generation of potentially pathogenic viruses by recombination between sequences of the vector used to introduce a transgene and related, but nonpathogenic, viruses that might be present in the same animal. These concerns are particularly acute for retroviral vectors. Retroviruses appear to be efficient vehicles for inserting transgenes into many species, including chickens, mice, cattle, fish, and shellfish, and might prove more successful than pronuclear injection of DNA in the generation of transgenic offspring. In many species, including chickens and pigs, there are endogenous proviruses (including the porcine endogenous viruses, PERVs, discussed below) that are competent for low-level replication in the host animal, but have no apparent pathogenic consequences. Endogenous proviruses are DNA sequences that were derived from infection of germline cells with a retrovirus and that are transmitted from parent to progeny like any normal gene. Their attenuation relative to their exogenous, pathogenic counterparts often is due to differences in transcriptional regulatory sequences in long terminal repeats (LTR's). As many vectors, such as the widely-used ones derived from murine leukemia virus (MLV), have LTR sequences derived from pathogenic viruses, the presence of both vector and endogenous provirus in all cells of a transgenic animal provides the potential for generating pathogenic recombinant viruses by straightforward and well understood mechanisms. Such concerns are particularly acute in chickens and pigs, where infectious proviruses very similar in sequence to those used for vectors are known to be present.

In mice, there is a well-studied model in which recombination between benign endogenous proviruses or endogenous proviruses, and infecting viruses early in the life of the animal, can cause a high incidence of lymphoma (nearly 100 percent in some mouse strains) 6 months later. Given this example, it is reasonable to expect that viruses of much greater pathogenicity are likely to arise in an animal when there is a possibility of recombination between vector and endogenous viral sequences. Similar concerns arise with the use of

vectors based on lentiviruses for the introduction of genes. Recombination of lentiviruses in circulation in domestic animal populations, such as Feline Immunodeficiency Virus (FIV) in cats and Bovine Immunodeficiency Virus (BIV) in cattle, with vectors based on Human Immunodeficiency Virus (HIV) is improbable due to the large genetic distance between them. However, vectors based on FIV and BIV are being developed and their use to introduce transgenes into the corresponding species would significantly increase the probability of generating more pathogenic recombinants.

4 Ensuring Confinement of Unwanted Animals

Although biopharm animals are not intended for consumption by humans or other animals, there are grounds for concern that adequate controls be in place to ensure that this does not happen without appropriate approval.

Since they do not contain the product of the introduced gene, there might be no strong reason to believe that eating or using products from transgene containing animals would pose a threat to human health; the possibility of such a threat combined with the lack of regulatory oversight for such uses argues strongly for confinement measures.

Although it has been stated that such animals will be too valuable to the owners to allow their misappropriation, the fact that the products of interest usually are produced only by lactating females means that half the transgene-containing animals essentially will be valueless, as will the females at the end of their period of useful production. "No takes," or animals generated from manipulated embryos, but culled because of lack appropriate expression of the transgene product (or lacking the transgene itself) also are inevitably generated in significant numbers during the production of transgenics. Thus, companies using biopharm animals are likely to seek approval for marketing food or rendered products from surplus animals, and the regulatory agencies will need to be ready to deal with such requests. Of greater concern is the possibility that surplus animals (and their carcasses) might, through inadvertence or theft, find their way into the food or rendering chain, or be used for breeding, thus allowing uncontrolled spread of the transgene into the general population. This would create a regulatory problem of dealing with unapproved transgenes after their release into the food chain-a problem analogous to that posed by the appearance in food products of Starlink, transgenic maize unapproved at the time for human consumption.

5 Biomedical applications

In contrast to genetic manipulation of farm animals for production traits, transgenic manipulation for the production of human pharmaceuticals or transplant organs generally is not intended to cause changes that have physiologic effects on the animals themselves. Thus, although unexpected and undesirable phenotypic effects still can occur as a result of gene insertion or cloning technology, there generally are fewer potential animal welfare concerns associated with the production of transgenic farm animals for biomedical purposes than for agricultural purposes.

5.1 Pharmaceuticals

Although there is a potential for producing pharmaceuticals in the eggs, blood, urine, or sperm of farm animals, the most common method is to produce transgenic cattle or goats that express the protein of interest in mammary tissue. The recombinant protein then is secreted in milk when the female lactates. This poses problems mainly when those proteins either are expressed in non-mammary tissues (so-called ectopic expression) or when they "leak" out of the mammary gland into the circulation. If the protein is active biologically in the species in which it is produced, it can cause pathologies and other severe systemic effects. Rigorous regulation of the expression of the transgene thus is necessary to ensure that the animal welfare consequences of milk-borne pharmaceutical production are minimized, but such regulation currently is difficult to achieve. However, even when a pharmaceutical is confined to the mammary tissue, the expression of particular proteins has been associated with premature lactational shutdown in goats and pigs. In pigs, there was evidence that the mammary tissue developed abnormally due to premature expression of the transgene, and that the condition of the mammary gland might have caused lactation to be painful. Similar concerns arise in the case of blood borne proteins and nutraceuticals (see below) if the products are produced at levels higher than the animal's normal physiologic levels.

5.2 Other Biomedical Applications

Farm animals might be genetically engineered for human biomedical applications other than xenotransplantation or the production of pharmaceuticals. Research is underway, for example, to produce a porcine model of cystic fibrosis, and there already are farm animal models for retinal degeneration and neurodegenerative disease. As genetic engineering techniques for farm animals improve-particularly such that single base coding changes that are typical of many human genetic diseases can be introduced, and the production and use of farm animal models becomes more economically feasible-it is likely that more models for disease research and toxicity testing will be developed. Discussion of the potential issues raised by these biomedical uses of farm animals is outside the scope of this report. However, the welfare implications will depend upon specific features of the model under study, including any unalleviated pain and suffering associated with the disease process itself, as well as the need for specialized husbandry and veterinary care requirements.

5.3 Xenotransplantation

Xenotransplantation differs from other uses of genetically engineered animals in that it has the potential to create something entirely new-permanent human–animal chimeras-in which cells of distantly-related species survive and function for long periods of time in the most intimate contact possible. Given its potential for alleviating human diseases due to irreversible tissue or organ failure (Table 2), and given the acute shortage of human organs for transplant, there are very active research programs underway, in both commercial and academic laboratories, to overcome the significant immunologic and physiologic barriers, and thereby to bring xenotransplantation into standard medical practice.

At present, the only animal under serious consideration as a xenotransplant donor is the pig. For regulatory purposes, human cells cultured *ex vivo* with the cells of any other animal, such as mouse cell lines, also are considered to be xenotransplants; co-cultivation with mouse cell lines has been used in the preparation of some cultured skin grafts as well as human stem cell lines. While nonhuman primates, such as the baboon, would seem to have physiologic and immunogenetic advantages such as the lack of a hyperacute immune response, their

scarcities as well as the difficulty of clearing them of adventitious infectious agents (as well as ethical concerns) render them impractical for further consideration.

Table 2: Applications of xenotransplantation

Indication	Transplant	Status
Organ Failure	Pig heart, kidney, liver, etc.	0
Acute liver failure	Extracorporeal perfusion	1
Diabetes	Pancreatic islets (or cells)	1
Parkinson's disease, Huntington's Disease, Focal epilepsy, Stroke	Neural tissue	1
Burn, Skin injury	Skin autograft (co-cultured with mouse cells)	2

Note:
0-No successful experience.
1-Some trials have been performed.
2-Successful trials have been performed.

The field of xenotransplantation covers a great many procedures, ranging from implantation of single cells to treat Parkinson's disease and tissues, such as pancreatic islets, to treat diabetes; extracorporeal use of intact organs, such as perfusion of patient blood through pig livers to provide short-term support in cases of liver failure; to transplantation of whole organs-heart, kidney, liver, and so on. While whole-organ xenotransplantation remains far in the future, development of the simpler modalities is underway, and hundreds of human subjects have received porcine cells or tissues as part of clinical trials in the United States, Russia, Israel, and many European countries. Given the nature of infectious disease issues, regulatory concerns are not limited to the United States alone, but extend to the international health community as well.

The development of xenotransplantation as a part of clinical practice promises great benefits in terms of making possible essentially infinite supplies of replacement tissues and organs where severe shortages exist today. This development naturally will entail both great potential benefit as well as considerable risk to the study participant, but such risk is not qualitatively different from that entailed in the development of any other new medical procedure and will not be considered further. The principal concern is that the uniquely close relationship created between recipient and host will allow novel opportunities for transmission of infectious disease, and possible creation of new disease agents in the process. While the history of close contact between humans and pigs is a very long one, and one would imagine that all possible transmission of infectious agents between the two species already would have been seen and thoroughly studied, it is possible that the "co-culture" environment of a transplant would be qualitatively different in ways that would allow different outcomes.

In an attempt to prevent hyperacute rejection of pig organs by humans, pigs have been made transgenic for the expression of human complement proteins, which are involved in regulation of the immune response. No phenotypic abnormalities have been reported in pigs as a result of the expression of transgenes for these human proteins, although, because the pigs are produced by microinjection, there are the usual inefficiencies in terms of the number

of embryos microinjected relative to the number of transgenic animals born. Research is underway to produce pigs that, in addition to carrying complement transgenes, have both copies of the gene encoding the enzyme that produces the antigen associated with rejection knocked out. The animal welfare implications of this genetic manipulation are unknown; however, the knockout, which causes changes in cellular carbohydrate structure, potentially could have deleterious physiologic effects on the animals and render them susceptible to infection with human viruses.

An important animal welfare concern related to xenotransplantation is the management and housing of pigs intended for use as organ sources. To minimize the potential for transmission of disease to human recipients, only specific pathogen free (SPF) pigs are used. SPF research animals are used in other contexts besides xenotransplantation, but their use raises several animal welfare issues. SPF pigs are born by hysterotomy or hysterectomy, and then are reared in isolators for 14 days before being placed in the source herd or in the xenotransplantation facility. The natural weaning age for pigs is about eight weeks (three to four weeks in commercial practice), and piglets subjected to extremely early weaning like this are known to develop abnormal behaviours. Older pigs intended for testing or organ donation might be housed in social isolation in unusually barren (i.e., easily sanitizable) environments. Pigs are extremely social animals that, when given the opportunity, will spend considerable time each day foraging, and that develop abnormal behaviors in confinement if not given the opportunity to root or build nests. In the United Kingdom, for organ-source pigs, while recognizing the importance of maintaining biosecure facilities, nevertheless recommends that such pigs be housed in stable social groups, and provided with environmental enrichment such as straw or other material suitable for manipulation. The Code requires justification if the animals' behavioral needs are to be compromised for a xenotransplantation protocol. There are no comparable standards for pigs intended for xenotransplantation in the U.S., and the lack of standardization of housing and care among U.S. facilities for these pigs is a source of concern.

Although there are many forms of environmental enrichment available those are suitable for laboratory-housed pigs, appropriate methods for organ-source pigs require development and evaluation.

5.3.1 Exogenous Infectious Agents

In general, bacteria and parasites that might cause problems readily can be excluded from source flocks, leaving viruses as the principal concern. As can be seen in Table 3, the number of viral agents that are of potential concern is very large. Not all of the viruses are on the list because of their potential to cause human disease; some would cause serious disease among the donor animals and others are sensitive indicators of breaks in biosecurity, and so forth. In principle, because all of these agents are horizontally (one animal to another) or vertically (mother to offspring) transmitted, they can be eliminated by proper management-proper containment, vaccination, close monitoring, culling, birth by Caesarian section, etc. In practice, elimination is going to prove a very difficult task, because the numbers of agents are very large and there is a lack of reliable assays for detecting many of them. Nevertheless, problems resulting from transmission of exogenous infectious agents are not qualitatively different from the present situation with human donors (allotransplantation), where infection with agents transmitted with the transplanted organ (such as Epstein–Barr virus and cytomegalovirus) is a major problem. In fact, it is anticipated that reduction in the risk of acute morbidity and mortality resulting from the transmission of infectious agents with transplanted organs will be a significant benefit of xenotransplantation.

Table 3: Exogenous pig viruses of concern in xenotransplantation

Family	Species	Category
Picornaviridae	Foot and mouth disease	
	Enterovirus 1 Talfan/Teschen	2, 5
	Enterovirus (other serogroups)	5
	Enterovirus swine vesicular disease	5
	Human enteroviruses	1
	Encephalomyocarditis	
	Rhinovirus	5
Caliciviridae	Enteric calicivirus	1
	Swine hepatitis E	1
Astroviridae	Porcine astrovirus	5
Togaviridae	Western encephalitis	1
	Eastern encephalitis	1
	Venezuelan encephalitis	1
	Getah	1
	Chikungunya	1
Flaviviridae	Japanese B encephalitis	1
	Louping Ill/TBE complex	1
	Wesslebron disease	1
	Apoi	2
	Dengue fever	1
	West Nile fever	1
	Classical swine fever (hog cholera)	5
	Bovine viral diarrhoea	5
	Border disease	5
Coronaviridae	Transmissible gastroenteritis	4, 5
	Porcine respiratory coronavirus	4, 5
	Epidemic diarrhea	4, 5
	Haemagglutinating encephalomyelitis	4, 5
	Porcine reproductive & respiratory disease syndrome	4, 5
	Porcine torovirus	5
Paramyxoviridae	Murine parainfluenza virus type 1 (Sendai)	2
	Parainfluenza 2	2
	Parainfluenza 3	2
	Blue eye disease	5
	Menangle	1
	Nipah	1
Poxviridae	Swinepox	5
	Vaccinia	2
	Cowpox	1, 5
	Orf/pseudocowpox	1, 5
Desoxyviridae	African swine fever	5

Note:
1=Zoonotic.
2=Replicates in human cells or weak evidence for zoonotic potential.
3=Might undergo abortive replication and possibly oncogenic replication.
4=Belongs to a family with evidence of frequent changes in host range or pathogenicity.
5=Undesirable as indicates a breakdown in biosecurity and/or might compromise health of the pigs.

5.3.2 Porcine Endogenous Retroviruses

PERVs present quite a different situation and level of concern because they are inherited as part of the host genome and, therefore, cannot be removed easily from donor animals. All pigs contain multiple (around 50) PERV proviruses in their genome, at least several of which encode infectious virus. PERVs are gammaretroviruses, closely related to MLV, which can be classified into three subtypes, A, B, and C, based on their envelope gene sequences. Subtypes A and B can infect many types of human cells in culture. Subtype C is much less infectious for humans. Most breeds of pig carry proviruses capable of yielding infectious virus of all three subtypes. Although most pigs carry about the same number of proviruses in their DNA, there is considerable diversity in location, implying that their insertion into the genome must have occurred rather recently (on an evolutionary time scale). Based on extensive experience with related endogenous proviruses of mice, it is highly likely that the majority of proviruses contain some sort of genetic defect, and that only a small number are responsible for release of infectious virus. Taken together with the polymorphism in the presence or absence of specific proviruses, it might well be possible to breed animals lacking infectious proviruses for use as xenotransplant donors.

PERVs have not yet been shown to cause disease (or even viremia) in pigs or any other species in which they have been detected. Nor has their presence been detected (by polymerase chain reaction, PCR, or serology) in more than 150 human recipients of pig cells or tissues, although a low level of infection of recipient cells can be observed in immunodeficient mice transplanted with porcine islets of Langerhans. Nevertheless, given the release of viruses infectious to human cells by many types of pig cells; the close similarity of these viruses to viruses known to cause cancer, immunodeficiency, and other diseases in mice and cats; the well-known adaptability and variability of retroviruses; and the example of the rapid worldwide spread of HIV and AIDS, there is serious concern that the novel association between pig and human tissues might create novel evolutionary opportunities for the virus, leading to the appearance of a new pathogen.

Although such a pathogen could have serious long-term adverse consequences for the transplant recipient, this issue is not an area of concern because it is far outweighed by the potential benefits of the transplant. The real issue of concern is that the xenotransplant setting might provide the opportunity for the virus to evolve into a pathogen that also could be transmitted from one individual to another efficiently enough to create a new epidemic disease.

It is virtually certain that many cells in the transplant would express infectious PERV following transplantation, and it events necessary for generation of pathogenic, transmissible viruses increasingly are unlikely, but on some unknown, arbitrary scale. Although the probability of inadvertent creation of a new epidemic generally is judged to be extremely small (particularly given the long history of intimate association between humans and pigs), it

cannot be ignored altogether. Current FDA policy is to permit xenotransplantation trials to proceed, but to require close monitoring of recipients, and (insofar as possible) of their contacts. Attempts also are being made to identify specific proviruses responsible for production of infectious virus and then to selectively breed them out of lines of animals to be used as transplant donors.

5.4 Food products from Non-genetically cloned animals

The cloning technologies of embryo splitting and blastomere nuclear transfer using embryo cells were introduced into dairy cattle breeding in the 1980s. Although not widely adopted, a total of 1,472 EMS cloned Holstein females was registered with the American Holstein Association through 2001and evaluated genetically for yield traits, meaning they produced calves and were milked commercially.

Yields of female EMS clones were greater than those of the Holstein population by 189 kg milk, 8 kg fat, and 7 kg protein, but slightly less than those of noncloned full siblings. The latter result might indicate an impact of the technology on performance or slightly different management of the two groups. Of 754 EMS cloned bulls registered and 143 evaluated by the U.S. Department of Agriculture (USDA) as sources of donor sperm, only 22 had noncloned full siblings. Results of the evaluations of the sires are not yet available. A total of 187 BNT cloned Holsteins (61 males and 126 females) were registered through 2001 (Norman et al., 2002); 74 had milk yield records, but only 11 had noncloned full siblings. The yields and milk composition of BNT clones exceeded those of the national herd average by 278 kg milk, 10 kg fat, and 10 kg protein, but were similar to those of their noncloned full siblings. Although existing data for EMS and BNT clones addresses the changes in milk yield and composition, they do not specifically address the food safety of their milk and meat products. Aside from a study on yearling Brangus bulls that compared body measurements and measures of carcass merit obtained from their steer clone-mates (Diles et.al., 1996), there are no published analytical studies of meat and milk composition comparing the products of cloned animals and full siblings evaluating in detail any unanticipated compositional differences, differences in protein quality, or nutrient bioavailability.

As the donor nuclei used to produce EMS clones are taken from embryonic cells, there is little if any genomic reprogramming needed to drive embryogenesis. However, blastomeres from embryos of more than eight cells, (i.e., from the stages typically used for BNT), must be reprogrammed upon NT, because they express a substantial number of genes, including paternal genes, that are not expressed by the oocyte nucleus. Indeed, the nucleus of the mature donor oocyte is transcriptionally quiescent and is associated with a different set of chromatin proteins (e.g., histones) compared to the recipient oocyte nucleus.

A similar array of gestational and postnatal abnormalities seen in somatic cell nuclear transfer also has been observed in BNT clones. To the degree that inadequate or otherwise different reprogramming relative to that occurring normally in gametic nuclei occurs in BNT, the composition of food products from NT animals might differ from that of ordinary animals. Although it is difficult to characterize the level of concern without specific data, it seems unlikely that there are changes in gene expression directly related to EMS and BNT cloning procedures that would rise nutritional or food safety concerns. Food products from BNT clones have been consumed by humans, with no apparent ill effects. Based on current scientific understanding, the committee regards products of EMS and BNT clones as posing a low level of food safety concern. Nevertheless, it would seem appropriate that the FDA use

available analytic tests to evaluate the composition of food products from animals that themselves result directly from BNT cloning procedures to verify that they fulfill existing standards for animal-derived food products. The products from the offspring of cloned animals were regarded as posing no food safety concern because the animals are the result of natural matings.

The cloning of animals from somatic cells is more recent. Limited sample size and health and production data, as well as rapidly changing cloning protocols, make it difficult to draw conclusions regarding the safety of milk, meat, or other products from somatic cell clones and their offspring. The key scientific issue is whether and to what degree the genomic reprogramming that occurs when a differentiated nucleus is placed into an enucleated egg and forced to drive the development of a clone might result in gene expression that raises food safety concerns. Differences in patterns of developmental gene expression in non-engineered individuals and somatic cell clones would be greatest during early development when reprogramming is incomplete. A number of datasets suggest that the health and wellbeing of neonatal and young somatic cell clones often are impaired relative to those of normal individuals. Direct effects of any abnormalities in patterns of gene expression on food safety are unknown. However, because stress from these developmental problems might result in shedding of pathogens in fecal material, resulting in a higher load of undesirable microbes on the carcass, the food safety of products, such as veal, from young somatic cell cloned animals, might indirectly present a food safety concern. As a somatic cell clone develops and nuclear reprogramming is completed, patterns of gene expression would approach those of a non-engineered individual. Indeed, the health and wellbeing of somatic cell clones approximated those of normal individuals as they advance into the juvenile stage. For example, somatic cell cloned cattle reportedly were physiologically, immunologically, and behaviorally normal, and exhibited puberty at the expected age, with high rates of conception upon artificial insemination. Two of these individuals have given birth to calves that seem phenotypically normal. There are to date no published comparative analytical data assessing the composition of meat and milk products of somatic cell clones, their offspring, and conventionally bred individuals. However, the committee found it difficult to characterize the level of concern without further supporting evidence regarding food product composition. Currently, there is no evidence that food products derived from adult somatic cell clones or their progeny pose a hazard (i.e., there is no evidence that they present a food safety concern).

5.5 Genetically engineered animals

A number of types of genetically engineered animals will be developed primarily for food, and others will be developed primarily for producing nonfood materials such as pharmaceuticals, vaccines, fibers, and other high value products. The principles for assessing the safety of food from genetically engineered animals are qualitatively the same as for non-engineered animals, but animals genetically engineered for non-food products might present additional concerns relating to the nature of the products that they generate. As for all foods or food products, those from genetically engineered animals should be evaluated for agents-chemical or biologic-which affect the safety of the food for the human consumer.

Animals used for xenotransplantation are not considered safe for human consumption and are excluded from the food chain by current regulations. Their exclusion is based primarily on concerns about persistent tissue residues of agents used to anaesthetize the animal before harvesting the tissues and organs. If there were any possibility that such animals might be rendered and considered for further processing into useful human food or

medical products, concerns about anesthetic residues would remain pertinent. If animals genetically engineered for xenotransplantation, but not used for that purpose, were presented for entry into the food chain, the food safety of such animals also would have to be evaluated based on protocols developed for evaluating other genetically engineered animals.

Animals might be genetically engineered to produce non-food products in their milk or eggs. Half of the genetically engineered population will be male, and will not be directly useful for production of heterologous proteins in, for example, milk or eggs. It is likely that companies producing such animals will seek early entry of males that are transgenic, but incapable of producing milk or eggs, into the food chain. In addition, companies might want to enter females that are "no takes", which do not express high levels of the product of interest, or that have reached the end of their productive lives, into the human food supply. The safety of food products from such animals that were culled from transgenic lines might present concerns.

Numerous experiments have shown that the level and specificity of transgene expression in an animal is predictable only to a limited extent, probably because all the factors affecting gene expression have not yet been identified. Transgenes might be expressed at a low level in various tissues in which the promoter is not expected to be active. Such ectopic expression might be due to genomic position effects attributable to the action of neighboring enhancer elements. In addition, ectopic expression might result from basal-level transcription at the site of integration. Recombinant proteins whose expression is driven by regulatory elements directing expression in mammary glands have been observed in the blood of transgenic animals during lactation. The presence of transgene products in blood might result from leakage of the mammary epithelium or from secretion at the apical side of mammary cells. For example, although the promoter from the whey acidic protein (WAP) gene has been used to direct expression of a transgene in mammary tissue, and some concentration of WAP normally is found in the blood of lactating animals. Hence, through bioactivity, allergenicity, or toxicity pathways, ectopic gene expression might directly affect the safety of food products derived from tissues, sexes, or life stages of transgenic animals where transgene expression is not expected. In some cases, recombinant proteins produced in milk have deleterious effects on mammary gland function or on the transgenic animals more generally. These effects might stem from ectopic expression of the transgene or from transfer of the recombinant proteins from mammary gland to blood. Animals with variable levels or ectopic expression of the transgene presumably will be identified in the development of the transgenic lines. Should products from such individuals be released to commercialization channels, they could pose a food safety concern unless the protein of concern is screened for and found absent. It is expected that well-established transgenic lines to be used in routine production will have been subjected to selection, and that concerns posed by unstable or ectopic gene expression will have been addressed to a large degree. Should pharmaceuticals or other biologically active proteins enter the food supply through products of such animals, associated food safety concern could be high. Additionally, the effects of transgene expression on animal wellbeing might indirectly affect the safety of food products derived from their tissues through stress-mediated mechanisms.

Expression of transgenes also might be intended to change the nutritional attributes or improve the safety of food products. For example, expression of transgenes in milk might optimize milk composition, add neutraceuticals to milk, or reduce the incidence of infectious disease. Several systems are being developed to reduce lactose concentration in milk.

Secretion of bovine-lactalbumin (an enzyme) in pig milk increased piglet growth, showing the potential for changing the nutritive value of milk.

Immunoglobulin A directed against viruses infecting the digestive tract might be expressed in milk and viral antigens activated by oral administration might be used to vaccinate humans and animals against viral disease.

Changes of these types raise a moderate level of food safety concern. Claims of nutritional attributes, safety, and efficacy of milk or other food products from transgenic animals must be demonstrated.

Animals might be developed to produce food products designed to fit special human dietary needs. Possible future products might include milk that lacks the most common allergenic protein, eggs that are lower in cholesterol, meat with enhanced vitamin content, or fat content modified in quality or quantity. The nutrient profiles of meat and animal products are well documented, and changes in this profile raise concerns. Changes might be unwanted by some consumers, and might add value for others. If these changed products were labeled to appeal to targeted consumers, and identifiable to those who have medical or other reasons to avoid such foods, they would be of low concern. Novel proteins also can be produced by genetic engineering.

Although proteins are necessary components of the human diet, they can exert undesirable effects, including: (1) allergenicity and hypersensitivity, (2) bioactivity, and (3) toxicity.

5.6 Other Bioactive Compounds

In some cases, the aim of genetic engineering is to enhance expression of an economically important trait e.g., growth rate or to improve resistance to disease e.g., mastitis. In others, animals will be engineered to express proteins of pharmaceutical interest. These applications involve the expression of biologically active proteins or polypeptides encoded by a transgene. The possibility exists that such molecules could retain their bioactivity after consumption, raising a food safety concern.

The bioactive product of a transgene, in most cases, will be a protein or, in some cases, a polypeptide. During digestion, proteins and larger polypeptides largely are broken down into small peptide fragments and amino acids by proteolytic enzymes in the digestive tract. Di- and tripeptides that are absorbed into digestive epithelial cells are broken down into amino acids by intracellular enzymes. Few intact, small peptides are absorbed into the bloodstream during digestion. Substantial degradation of the intact protein effectively destroys its original bioactivity, because that bioactivity depends on the integrity of at least a portion of the protein or peptide. Allergenicity might, of course, remain a problem for sensitive individuals. Many food allergens are absorbed into the bloodstream. While most intact proteins generally are not absorbed into the bloodstream of healthy adults with an intact, properly functioning digestive system, absorption might occur in individuals, whose digestive epithelium has been compromised by disease or injury, possibly posing allergenic response. In such cases, the normal array of digestive enzymes might be absent, or the integrity of the epithelium as a barrier might be compromised.

Gastroenteritis, for example, can reduce the secretion of digestive enzymes and cause the breakdown of digestive epithelium, resulting in the passage of intact proteins and peptides

into the bloodstream. A food safety concern thus arises when individuals whose digestive system has been compromised by disease, injury, or advanced age ingest foods containing bioactive proteins or peptides.

The digestive epithelium of newborn infants permits the transient absorption of whole proteins or large protein fragments until closure of the gut epithelium occurs. Closure is facilitated by breastfeeding and delayed in infants that are formula fed. The timing of the closure might range from weeks to months, depending on dietary factors. Before closure, a wide variety of intact proteins might cross the digestive epithelium by a non-selective mechanism and enter the bloodstream. Thus, consumption of food (especially milk) containing bioactive proteins or peptides could result in the transfer of such molecules into the bloodstream of newborn infants. This possibility raises a concern regarding recombinant bioactive molecules present in milk used in infant formulas.

Bioactive peptides and proteins also might exert their effects in the digestive system, before absorption. For example, recombinant human bile-salt stimulated lipase (BSSL) has been expressed in the milk of transgenic sheep; this protein is intended for oral administration as a therapeutic agent for treating patients suffering from pancreatitis. Consumption of food products (i.e., milk and meat) from animals expressing bioactive molecules such as BSSL could alter digestion in otherwise healthy individuals, and presents a food safety concern. Lysostaphin, a bactericidal protein expressed by certain bacteria, has been expressed in murine milk, where it reduced mastitis caused by *Staphylococcus aureus*. Transgenic cattle expressing lysostaphin in milk have been generated with the intent of reducing mastitis in that species. Similarly, transgenic fish is produced to express the hybrid antimicrobial peptide cecropin-melittin for control of fish pathogens. Preliminary studies using injections demonstrated the effectiveness of the antimicrobial peptide to protect fish against infections and suggested that the strategy of overexpressing the peptides in transgenic fish might provide a method of decreasing bacterial disease problems in fish. Milk containing lysostaphin or fish expressing cecropin-melittin could alter the balance of digestive tract flora of consumers of these products; in addition, widespread use of such antimicrobial agents also could foster the emergence of lysostaphin-resistant strains of pathogenic *S.aureus* or *Vibrio anguillarum*. Thus, food products containing antimicrobial proteins might present a food safety concern in view of their potential to alter the balance of consumers' intestinal flora, and might foster the evolution of microbial strains resistant to specific agents.

Many genetically engineered fish and shellfish express an introduced growth hormone (GH) gene-most often a fish GH gene to promote rapid growth. Hence, it is particularly important to make sure that such a transgene product has no biologic activity in humans or animals that consume fish or shellfish expressing such a transgene. The food safety of GH proteins was evaluated when administration of recombinant bovine GH (rbGH). The FDA cited data showing that non-primate GH proteins are not biologically active in humans; nor are fragments of the GH molecule, nor insulin-like growth factors secreted by the host in response to GH administration. Neither bovine GH nor bovine insulin-like growth factor I (IGFI) was orally active in rats, a species responsive to parenterally administered bovine GH. The FDA also cited studies showing that bovine, ovine, whale, and porcine growth hormones are not biologically active in humans, which suggests that piscine growth hormones are unlikely to be biologically active in humans.

The degree to which full-term human infants absorb intact proteins is equivocal; FDA cited studies showing that concentrations of IGF-I in milk of rbGH-treated cows was within

the physiologic range in human breast milk, and IGF-I is denatured under conditions used to process cow's milk for infant formula. In the unlikely case that products of GH-transgenic fish or shellfish would be fed to human infants, cooking would denature active GH and IGF-I molecules. Against the background of the discussion above, the committee regards the likelihood that a bioactive product poses a hazard will vary among gene products, food products, and consumers, in various cases posing a low to moderate level of food safety concern. For a susceptible individual, however, such a hazard could have severe consequences (Table 4).

Table 4: Examples of Bioreactors

- Naked human Hb from pigs
- Human lactoferrin in cows' milk
- Alpha-1-antitrypsin in sheep
- HGH in mouse urine (uroplakin promoters)
- Human antibodies in mice (H and L chain tgenics → hybridomas)
- CfTCR in goats
- Tissue plasminogen activator (TPA) in goats
- Human antithrombin III in goats
- Malaria antigens in goats (vaccine)
- Alpha-glucosidase in rabbits (Pompe's disease

6 Toxicity

Many toxins are well studied and genes for known toxins would not be transferred purposefully into a food animal. As noted earlier, genetic engineering will have the potential of introducing novel proteins expressed in food animals. Because proteins generally are broken down in the digestive system into common amino acids, the direct toxicity of proteins (beyond the possibilities of allergenicity and bioactivity discussed above) is unusual and generally of low food safety concern. Purposefully expressed proteins that remain intact or otherwise pose a potential safety concern presumably will be fully evaluated in the pre-market review and approval process, and thus pose a relatively low concern.

Of greater concern to the committee with respect to possible toxicity are the unintended and unanticipated effects and byproducts of the genetic engineering of a food animal, including but not limited to these novel proteins. For example, the engineering could alter a metabolic process that then results in a toxic metabolite being present in edible tissue. The question, as suggested is whether edible products of genetically engineered animals have been screened adequately to detect the presence of unanticipated compositional changes that might introduce toxicity. Assuming that adequate analytic methods and screening protocols exist (an issue that the committee did not examine during its deliberations) and are applied, the possibility of such toxicity poses a low level of food safety concern to the committee.

7 Farming

If genetic technology becomes more efficient and affordable, the primary farming applications of transgenesis and cloning likely will be to produce animals with increased growth, improved feed conversion, leaner meat, increased muscle mass, improved wool

quality, improved disease resistance, and increased reproductive potential. The technology also can be used to produce food of improved nutritional quality (nutraceuticals) or appeal. The primary difference between traditional breeding and genetic engineering is the speed at which change typically occurs (although naturally occurring mutations and recombination events also can cause rapid and dramatic change), and the single-gene nature of genetically engineered change.

Traditional methods of selection are more likely to be subject to the checks and balances imposed by natural selection. Many related and apparently unrelated traits are correlated genetically; thus, selective breeding involves selecting for a whole phenotype rather than a single gene product. Because most production and behavioural traits in livestock are polygenic and our understanding of livestock genomes is poor, few traits can reliably and predictably be engineered or introduced by manipulating only one gene. For this reason, the production of a line of transgenics will require generations of selective breeding after the introduction of gene constructs into the founder generation to ensure that animals display the desired phenotype with few or no undesirable side effects. However, it is clear that serious welfare problems also have resulted from traditional breeding techniques. Broiler chickens are a case in point. Breeding for increased growth has led to serious physical disabilities, including skeletal and cardiovascular weakness. A large percentage of broilers have gait abnormalities and these might be painful, making it difficult for the birds to walk to feeders and waterers. In addition, broiler hens must be severely feed restricted to prevent obesity, and this feed restriction is associated with extreme hunger and a variety of behavioral problems, including problems with mating behaviour and hyperaggressiveness. Traditional selection of pigs for increased leanness has led to increased excitability during handling and selection for high reproductive rates (either by shortening the interval between births or increasing the number of offspring born) or increased lactation also has led to welfare problems.

8 Potential animal welfare benefits

Genetic engineering certainly has the potential to improve the welfare of farm animals. Decreasing mortality and morbidity by increasing resistance to diseases or parasites, or decreasing responses to ingestion of toxic plants, are obvious examples of welfare benefits, and an area in which some transgenic research is focused. It also has been pointed out that transgenic animals might receive a higher standard of care than nontransgenic animals because of their greater economic value. Cloning could be used as a strategy for breed preservation to maintain genes that are important for adaptation and resistance to disease, but equally could result in a further narrowing of the gene pool, with possibly deleterious effects on animal health.

Improving disease resistance to decrease pain and suffering is an application of transgenic technology that has clear animal welfare benefits. But it should be stressed that animal welfare is multifaceted, and this needs to be taken into account when assessing welfare impacts of the application of any technology-not just biotechnology. Important elements of animal welfare include freedom from disease, pain, or distress; physiologic normality; and the opportunity to perform normal behaviours. While reducing disease clearly is beneficial, if this also permits animals to be confined more closely, and thus decreases the opportunity for them to perform their normal behaviors, then the net effect on welfare could be negative.

Chapter 18
Piscitechnology and aquatic biotechnology

1. Fisheries

Since the late 1980's, capture fisheries landings have been levelling off at 80-85 million metric tons (mmt) and this productivity is presently regarded as near the upper limit. In the last decade, at least 20 major fisheries have collapsed around the world which was quite closely monitored and supposedly rationally managed. As of 1995, 6% of the target fish stocks were already depleted, 16% was overexploited and 44 % fully to heavily exploit. This emerging awareness of the vulnerability of marine resources throws discredit on the complacent view that sea-fisheries are essentially inexhaustible because compensatory resilience of target fish stocks would be ensured by their great fecundity and vagility.

With a growing concern about ecosystemic impacts by fisheries (overfishing, destruction of benthic communities by dredging, trawling and other disruptive fishing practices, perturbation of food webs by the non-selective removal of target animals with consequent massive wastage of nonmarketable species, etc.) as well as about environmental impacts on fisheries (chemical pollution from coastal runoffs, eutrophication and algal blooms, oil spills, marine habitat fragmentation and degradation, introduction of foreign species, man-induced global changes on weather and ocean physics, etc.), there are discouraging perspectives about the capability of capture fisheries to satisfy the anticipated increase in the demand for seafood by the expanding human population. Actually, improvements in fishery management are expected to rely on more precautionary approachest o fishing effort and harvesting pressure, implementation of marine reserves and temporary "notake" zones, enforcement of moratoria, adoption of more selective fishing gears and other remedial measures that are likely to sacrifice short-term economic gains in favour of more sustainable, though diminished catches.

In the Mediterranean Sea, the situation is particularly critical owing to its relatively small extension, moderate overall bioproductivity and great number of countries with gravitating fisheries. A regulation from the European Community enforces technical measures to preserve the Mediterranean fishing resources. These measures that establish the minimal net meshes and fish sizes for fishing activity should be complemented in the future by co-operative agreements among all interested nations about more effective approaches for fish stock assessment and conservation.

2. General technology for Fish propagation

One prerequisite of intensive fish culture technology is to have healthy fish seed in the required amount available. This can be realized only with preliminary propagation planning. Besides natural and semi-artificial propagation, artificial propagation carried out in a well-equipped hatchery is the up-to-date way in which the necessary quantity of fry can be produced.

2.1 Culture and Management of Brood Stock

A basic precondition of fish propagation is to have sexually mature male and female fish producing milt and eggs. Breeders of fish species which cannot be cultured or kept

domestically, are collected before spawning from rivers or lakes, their milt or eggs are stripped and then they are released again. These species are sturgeons (mullets) mugils (Mugilidae), and salmonids (Salmonidae).

The brood stock of cultured fish species are sorted out with aimed selection and cultured in fish ponds up to sexual maturity. The males and females of breeders are cultured in the same fish pond while they are young. Then, when sexually mature they are selected and cultured in separate fish ponds.

2.2 Environmental Conditions for Culturing Breeders

When culturing breeders, the following environmental conditions should be ensured considering the specific demands of the respective species: Suitable water and pond, temperature, oxygen and feed light/photoperiod.

During culturing of breeders, the water quality should meet the same requirement as during routine fish culture.

For species with preference for still water (e.g., cyprinids, catfish, tilapia) earthen or concrete-walled fish ponds should be constructed. For rheophyl species (e.g., trout) flow-through systems, canals, or tanks with circulating water should be provided.

The temperature, as an environmental factor, has an important role in fish culture. Fish are a polikilotherm animal, which means that their body temperature follows the surrounding temperature, but in case of increased muscular work and metabolism it can be somewhat higher. Fish can be warm water or coldwater species. Coldwater fish are salmonids and trout. Their optimal temperature range is 10-16°C and their oxygen demand is high.

Warm water fish are the cyprinids like common carp, Chinese carp, Indian carps and from other families the tilapia, milkfish, mullet and the American catfish. Their optimal temperature range is 18-26°C.

There are some species "in-between", they reproduce in cold water, but later can live in warm waters too. Such are pike and pike-perch. The development can be greatly influenced by water temperature. Feeding at low temperature can be normal, but the conversion of the feed, and consequently the growth, especially sexual maturation can be considerably slower. The duration of sexual maturation also depends, first of all, on the environmental temperature, with most fish species, thus there is a characteristic total temperature value for each species which is necessary for gametogenesis.

In the case of carp, from hatching to the first reproduction, 10-12 000 degree days of heat is necessary. This quantity of heat under a tropical climate is ensured in l-l.5 years while in Middle Europe in 3.5-4 years.

1 600-2 000 degree days is necessary in the proper temperature range for the oogenesis of matured carps between two propagations. If the temperature is lower than that, considering the days with temperature higher than 17°C only, it is 2 500-2 700 degree days. The minimum temperature which is necessary for propagation of carps is 17°C, below this value the gametogenesis is very slow.

Optimal oxygen content of water is very important especially in active cytogenetic processes. Development of gametes and complete conversion of feed are all oxygen consuming processes. In case of oxygen deficiency the gematogenesis slows down,

development is inhibited and if it is prolonged the resorption of gametes starts. Therefore, the proper oxygen supply is a crucial point of brood stock culture.

Feeding of breeders, especially in the nutriment accumulating phase of oogenesis requires feed of specific composition. Amino acids, carbohydrates, fats, vitamins, and minerals are available for the fish via natural feeding. But at high stocking density under controlled culturing conditions these must be provided through artificial feed. It is also important that the breeders can get the type of feed characteristic of their species, i.e., herbivorous fish should get nutriment of plant origin, and carnivorous should get nutriment of animal origin.

Light/photoperiod has a significant role in the reproductive processes of several fish species.

Stress factors, and the sensitivity of the cultured breeders to these factors also should be kept in mind. Handling, transportation, enclosure which all differs from their normal lives, are stress inducing factors, and may affect their condition. This may hinder the last phase of maturation and ovulation. Therefore, conditions should be established for the breeders where all these stress factors can be minimized.

- Stocking density must be optimal, thus ensuring a supply of natural food
- Water supply, flow rate, temperature and oxygen content should meet the requirements of the fish;
- Breeders during spawning are kept in smaller size fish ponds or concrete tanks. Their filling or draining should be efficient because the fish must be caught very often;
- Breeders can be transported in tanks only, maintaining the proper oxygen content by spraying liquid oxygen;
- Case of timid fish (e.g., Chinese carps) sedatives can be added to the water;
- When handling is necessary, the seine can be applied. Manual transportation of fish needs skill, so well-trained fishermen should do it. If the breeder with eggs is dropped it may lose the eggs, the ovary inflames and in most cases the fish dies.

3. Practical technologies of the artificial propagation of cultivated Fish species

3.1 Common carp
Species: Common carp (*Cyprinus carpio* L.)
Origin: China, South Asia, Europe
Natural habitat: Rivers, lakes, fish ponds
Feeding and nutrition: Zooplankton, water insects and their larvae, bottom inhabiting organisms, grains and granular and pellet foods

Weight, at age intervals: (Europe only)

Year	Grams
1	20 - 100
2	200 - 500
3	800 - 2 500
4	3 000 - 4 000
5	4 000 - 5 000

3.1.1 Reproduction

In a natural environment, the fish spawn in groups, in lakes and slow moving rivers, at a water temperature of 18 to 22°C. The egg attaches itself to vegetation and hatches within three days. In extensive cultivation they are spawned in small ponds.

3.1.2 Economic characerization

Rapid growth, High degree of fecundity, High meat quality, though containing intermuscular bone and very suitable to warmwater polyculture Good sport fish.

3.2 Chinese Herbivorous Fishes

Species:
Silver Carp (*Hypophthalmichthys molitrix* Val.)(SC)
Bighead Carp(*Aristichthys nobilis*)
Grass Carp (*Ctenopharyngodon idella* Val.) (GC)

Origin: Southeast Asia
Natural habitat: Large rivers, warm water, lakes, fish ponds
Feeding and nutrition:
Silver Carp (SC) - phytoplankton diet
Bighead Carp (BC) - zooplankton diet
Grass Carp (GC) - aquatic macrophyte diet

Size at ages in grams

Years	SC	BC	GC
1	Oct-15	15 - 30	Oct-20
2	100 - 200	150 - 300	150 - 250
3	500 - 800	800 - 1 500	800 - 1 500
4	1 500 - 2 000	3 000 - 4 000	2 500 - 3 000
5	3 000 - 4 000	5 000 - 6 000	4 500 - 5 000
6	4 500 - 5 000	7 000 - 8 000	6 000 - 7 000

3.2.1 Reproduction

- In slowly flowing warm rivers they spawn in groups.
- In cultured conditions, spawning occurs only artificially.
- Spawning temperature, 22-26°C.
- The eggs are pelagic.

3.2.2 Economic characterization

- Good quality of meat.
- Good sport fishing (GC).
- In polyculture they utilize the primary and secondary production of the water directly.- GC controls the macrophytes in lakes and channels.

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3.2.3 Artificial propagation

The stock material is kept in a special pond and the stocking rate per hectare (ha).

Age by spawning in years

	Female	Male
SC	05-06	04-05
BC	07-08	06-07
GC	06-07	04-05

3.2.4 Sexual differentiation

The first hard rays of the pectoral fins of the male are sharp while those of the female are smooth.

3.3 European Catfish

Species: European catfish (Sheatfish, *Silurus glanis* L.)
Origin: a typically European species
Natural habitat: Rivers, lakes, artificial ponds
Feeding: carnivorous predator will also eat carrion. Well suited to artificial feeds

Weight, at age intervals:

Year	Grams
1	20 - 100
2	200 - 400
3	500 - 2 000
4	3 000 - 4 000
5	5 000 - 6 000
6	7 000 - 10 000

3.3.1 Reproduction

In natural circumstances the fish uncover the roots of aquatic vegetation, depositing the eggs among these roots. The eggs cohere into a lump. After hatching, the larvae remain hidden here. Six to eight days later, the fry emerge swimming and depart in search of food.

In rearing ponds, they spawn on "artificial nests", which are then removed to the hatchery. May be artificially propagated in hatcheries. Required temperature for reproduction is 22 - 24°C.

3.3.2 Economic characterization

-Rapid growth;
-Good quality meat, with no intramuscular bone;
-Eats undesirable small fish;
-Good sport fish;
- Well suited to polyculture.

Distinguishing sexual characteristics

- The frontal portion of the head, about the mouth, is angular in the male, while rotund in the female;
- The genital papilla of the male is pointed, that of the female blunt and rounded.

3.4 Pike Perch

Species: Pike perch (*Stizostedion lucioperca* L.)

Habitat: Prefers oxygen rich waters. It lives in rivers, lakes, reservoirs and fish ponds

Feeding: It is a carnivorous predator, feeding mainly on small fish

Weight at age intervals

Year	Grams
1	10-20
2	100 - 200
3	400 - 700
4	800 - 1 200

3.4.1 Reproduction

In its natural environment, it uncovers the roots of aquatic vegetation, depositing the ova among them. The male then fertilizes the eggs. It spawns in pairs, and guards the ova until they hatch. Temperature at spawning: 14°C.

3.4.2 Economic characterization

Its meat is outstanding, and contains no intramuscular bone.

- Good sport fish;

- In polyculture, it consumes small fish of no value;

- It does not withstand well the handling necessary in harvesting and transportation, thus requiring extraordinarily careful treatment.

3.4.3 Propagation

Care of the brood stock

- In the autumn the brood stock is harvested from natural waters or fish ponds;
-The stock is placed in wintering ponds without segregation by sex;
-Wintering space requirement is 3 to 4 m^2 per fish;
- Small fish, at the rate of 20 to 30% of the brood stock by weight, are placed in the pond;
- Continuous water circulation must be ensured in the wintering ponds.

3.4.4 Sexual differentiation

- The female's belly is full, rounded and its general area is white in color;
- The male's belly is flatter, its area gray.

3.5 Semi-Artificial Propagation of Indian Carps

Species:

Catla catla (Hamilton) (Catla)

Labeo rohita (Hamilton) (Rohu)

Cirrhinus mrigala (Hamilton) (Mrigal)

Origin: India, Pakistan, Bangladesh, Burma

Natural habitat: Rivers, lakes, fish ponds

Feeding:

Catla catla - zooplankton, phytoplankton in surface strata

Labeo rohita - zooplankton, along the whole water column

Cirrhinus mrigala - omnivorous bottom feeder.

Age dependent size of fish

Year	Catla mm	Rohu mm	Mrigal mm
1		250-380	290
2		280-510	511
3		415-660	670
4		510-750	797
5		550-810	858

3.5.1 Propagation
-Spawning in rivers during monsoon rains;
-Spawning can be induced semi-artificially;
-Optimal temperature of spawning is 26-31°C;
- Eggs are floating, not sticky.

3.5.2 Economic features
Applicable in intensive polyculture technologies and the meat is tasty, important food.

3.5.3 Way of distinguishing male or female spawners
The belly of female is swollen, genital organ is red, upon light pressure the male produces white milky drops. The hard ray of pectoral fin is rough with the male, and smooth with the female.

3.6 Artificial propagation

Rearing and keeping of the brood stock: the young brood stock is selected from the offspring of parents of known quality. The selection criteria are: Rapid growth, Good viability, High food conversion rate, Low fat content and resistance to disease. The brood stock is reared in individual ponds, segregating male and female. The stocking density of the rearing ponds is 500 to 1 000 adults per hectare.

Feed is an artificial pellet, containing 20 to 25 percent of protein, of which 15 to 18 percent is animal protein; fats 3 to 4 percent; vitamin premix 2 percent; mineral pre-mix 1 percent. Vitamins A and E are critical. For two months before propagation the daily feed should contain 5 to 10 percent of raw ground meat or hard boiled eggs. Feed quantity is 2 to 5 percent of body weight, per day.

Sexually mature fish measure 35 to 70 cm and weigh 2500 to 10000 g. The fish achieve sexual maturity in Europe: female 3 to 4 years, male 2 to 3 years. In tropical climates: female 1 to 2 years, male 1 year.

The female has a large belly. The male, if lightly squeezed around the belly will emit white milt.

Handling of the spawners: The spawners should be moved into the hatchery one day before propagation; The spawners should be transported in containers made of inorganic, non metallic materials; The male and female spawners should continue to be segregated in the hatchery, in plastic or concrete tanks; Space requirement is 0.5 to 1m²/adult; Tanks should be 5 to 10 m², 1 to 1.2 m deep; Water quantity required is 4 to 6 litres, per fish, per minute; Oxygen content of the water should be 6 to 8 mg/l; Water temperature should be 20 to 22°C; Before handling, the fish may be tranquilized; The tranquilizer recommended is MS 222 (Sandoz), in a 1:10 000 dilution and after 5 to 10 minutes in the above solution, the fish should be moved into fresh water of high oxygen content, otherwise they will perish.

3.7 Hypophysation

To induce ovulation in the female and milt production in the male, hormone of the carp's pituitary gland is used. The dosage used in the hypophysation of the female is 4 to 4.5 mg per kg of body weight. The dosage for the male is 3 mg per kg of body weight. - The dried pituitary gland is pulverized and dissolved in a 0.65 percent solution of salt (NaCl); 2 ml salt solution per fish is used. The hypophysation of the female should be done in two stages; ten percent of the dosage is given 24 hours before the removal of eggs; 90 percent of the dosage is given 12 to 14 hours before the removal of eggs, if the water temperature is 21 to 22°C (240-260 degree hours). The injection is done with a fine needle, into the muscle of the back. Before withdrawal of the needle, the locus of the injection is lightly massaged, to avoid leakage of the solution injected. Concurrent with the second injection, the opening of the oviduct is sewn up to prevent the loss of mature eggs during the period of ovulation. The suture is done with surgical implements. The male is to be injected 24 hours before the desired time of milt discharge. It is imperative that the fish in the hatchery have absolutely quiet surroundings.

The removal of eggs and milt, fertilization. One hour before removal of the eggs, one or two males are placed among the females. At the onset of ovulation, the male and female fish will swim alongside one another, flapping forcefully. One half hour should elapse to assure that the full quantity of eggs will have detached itself from the walls of the ovary. After this time, the female fish are tranquilized. The sutures are removed from the opening of the oviduct. The belly of the fish should be wiped dry with a cloth, to prevent wetting the eggs which will rapidly lose their fertility if mixed with water. The eggs are released into 2 litre plastic dishes; if necessary, light pressure may be applied to the belly to make the roe flow out. The milt is pressed out of the male with light pressure into test tubes. The eggs should be fertilized immediately upon their removal from the female. Ten to twenty ml milt is used to one litre of eggs. Each batch of eggs should be fertilized with milt from no less than three males, because the sperm of any male may occasionally be inactive. The eggs and milt are thoroughly mixed (without the addition of any water), so that all eggs are reached by the milt. The mixing should be done with a plastic spoon. Fertilization is promoted by the use of a fertilizing solution. The ingredients of the solution are: water, 1 litre salt, 4g urea, 3g ($CO/NH_2/2$), temperature of the solution should be 20 to 22°C. The fertilizing solution has a two-fold effect, it prevents the adhesion of the eggs and it activates the sperm. 100 ml of the solution is added to 1 litre of roe and immediately mixed with it; the mixing action should be continuous at two minute intervals, another 100 ml of the solution is added to the mixture. After ten minutes, the fertilizing solution is poured off, fresh fertilizing solution is added, 2 litre solutions to 1 litre of eggs. The eggs, together with the fertilizing solution, are then poured into a plastic container of 15 to 20 litre capacity, the eggs will swell to 4 to 5 times their original volume within one hour. For one hour, the fertilizing solution should be exchanged at ten minute intervals, to continue to dissolve and remove the adhesive of the eggs. Mixing should be done intermittently and gently whether

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manually or mechanically. After one hour of the above process the eggs should be treated with a tannic acid solution, to dissolve the adhesives coagulated during the previous treatment and still present on the surface of the eggs. The tannic solution consists of 1.5 gram tannin dissolved in 1 litre water, treatment with the tannic solution is preceded by pouring off the fertilizing solution and one to two litres of tannic solution is added to the eggs and immediately mixed in. After ten seconds, water is added to dilute the tannic solution. The solution is then immediately poured off. The eggs should then be rinsed 3 or 4 times with ample quantities of water and after rinsing the eggs are placed in hatching jars.

3.8 Hatching

Water temperature during hatching should be 20 to 22°C and one litre of hatching jar volume is needed for each 20000 eggs whereas the volume of 20000 swollen eggs is 200 ml. The eggs are a risky process, because the segmentation of the ovum is already started and strong jolting will destroy it. So, the eggs are placed in the water-filled hatching jar with the aid of a funnel, which is equipped with a length of hose, to assure that they will exit from it under water. In the first ten hours, the hatching jar is to have a moderate flow of water: assuming a 10 litre jar, the rate would be 0.8 to 1 l/min. After ten hours, the flow should be increased, because the oxygen need of the ova is on the rise, to a rate of 1.5 to 2.5 l/min and the eggs will be drifting loosely on the bottom of the hatching jar.

Four to five hours before hatching, the embryos' need of oxygen is considerable; therefore, the flow rate should be increased to 2.5 to 3 l/min. On the second day of the hatching process, the eggs should be treated with malachite green to prevent the generation of fungus. Malachite green is added to the water in the hatching jar in a quantity to give a 1:200 000 solution and let the solution stand for 5 minutes. After 5 minutes, the water flow is restored and the malachite solution will thereby be slowly washed out. Hatching of the larvae may be expected on the third day. After the first few larvae have hatched, the process should be artificially accelerated which is accomplished by shutting off the flow of water for 10 minutes. The lack of oxygen agitates the embryos, and due to their movements the shell will split. After 10 minutes, the water flow is restored and hatching will start on a large scale. The hatched larvae are transferred, together with the water, into larvae containers.

Maintenance of the larvae

The implements used in the maintenance of larvae may be any one of the following:
1. Cylindrical containers, conical at the lower end, of 50 to 150 litre capacity, of glass, plastic or fibreglass, 2. Circular flow raceways of concrete or plastic, 3. Cages made of nylon cloth (screen).

1. Cylinders

In the containers 2 000 larvae per litre of water can be maintained. Water should enter at the bottom and flow out at the top of the container. The area of the overflow screen should be 10 cm^2/litre of capacity. The flow rate should be such that the larvae are kept in suspension but not washed onto the overflow screen. The screen should be cleaned at intervals, as it will be stopped up with remains of shells. The larvae are kept in these containers only until their first intake of feed (3 to 4 days), after which they are transferred to rearing ponds. The above process is the most up-to-date and most hygienic method of keeping the larvae.

2. Raceways

The raceways, of plastic or concrete, should be of 1 to 2 m^3 capacity, the depth being 60 to 80 cm. Water enters the raceway through perforated tubes placed vertically at two opposite sides, to assure a circular flow. The rate of flow should be such that it produces a constant

slow motion of the water. The overflow tube is placed in the centre, vertically. The overflow screen, of nylon, should have 0.8 mm openings. Surface area of the screen should be 8 to 10 cm^2/litre of flow. The oxygen content of the water is increased by surface spraying. The raceway should be stocked at the rate of 1 000 hatchlings per litre of water. The advantage of this method is that the larvae can be kept for several days beyond the first feeding, if suitable nourishment can be assured.

3. Cages

The size is 70×40×30 cm, frame should be made of aluminium or plastic tubing and Screening made of nylon, 0.8 mm opening. The cages are kept in plastic or concrete tanks. An upward flow of water through the cages is needed which keeps the larvae in motion. This is affected by laying tubing, perforated at the top, on the tank bottom. The bottom of the cage should be cleaned the day after hatching to remove shells and unhatched larvae, which will start to decompose thus causing oxygen deficiency. The bottom and sides of the cage should be cleaned with a brush from the outside at 6-hour intervals to assure free passage of water into the cage. The rate of flow in the tanks should be 4 to 5 l/min/cage.To increase the oxygen content of the water, a fine surface spraying should be used.

Feeding and transportation of the brood

The mouth and gills of the carp larva develop in 3 to 4 days. The carp's larval stage ends with the first intake of food, at this point it becomes fry. At this stage the yolk sac will have been largely absorbed; the hatchlings will rise to the surface of the water, fill the swimming bladders with air and begin to swim horizontally and the first feeding is given. The feed is yolk of hardboiled egg (hen's or duck's egg) mixed in a blender with water; 100 ml water per one yolk is used and is given one to two ml per 100000 fish. As soon as the hatchlings begin to feed, they should be transferred to ponds. The 4-day old feeding fry are transported in plastic bags containing water and oxygen. In 20 litres water plus 30 litres oxygen, at 20°C water temperature, 100 000 brood may be kept up to 5 hours; at 15°C, 200 000 brood may be kept up to 5 hours. If chilled for transport, the temperature of brood and water should be lowered gradually. The plastic bags, when filled, should be as firm as well filled balloons. The bags must be well tied, to prevent escape of the oxygen. To avoid rupture, the bags should be placed in cardboard boxes. The 4-day old larva measure 6 to 7 mm in length.

4. Aquaculture

While many capture fisheries are facing declining or stagnant harvests, there is a widespread confidence that a growing portion of the necessary production to secure future food fish availability will eventually be supplied by aquaculture. This expectation is based on the impressive figures of total world aquaculture growth in the past decade, with a doubling of landings from 10.4 mmt in 1984 to 22.6 mmt in 1993. In this scenario, the production figures of marine fish culture in the Mediterranean Basin are comparatively small, but their increment is nonetheless striking as seabass/seabream farming has increased six-fold in the period 1990-1995 reaching about 42,000 tons produced by almost 0 intensive enterprises.

On a global scale, aquaculture is currently one of the fastest growing food production systems, increasing at an annual rate of 9% compared to 2.8% for total livestock meat production. It is estimated that to maintain current levels of food fish consumption, global aquaculture production should be doubled by the year 2020 and trebled by 2040. Whether this anticipation is an achievable goal, however, is still an open question. Aquaculture is an extremely heterogeneous industry devoted to the farming of a multitude of animal and plant

species and characterized by a marked technological polarization. In developing countries (which account for 85% and 86.7% of total and finfish global aquaculture production, respectively), traditional low cost, low-stocking density, subsistence-oriented polyculture technologies are mainly used, whereas in developed countries high-cost, high-stocking density, profitoriented monoculture technologies prevail.

Although both farming systems are at present economically viable in their respective areas, increasing competition for land and water as well as broadening of international food fish trading are promoting a trend towards greater intensification of farming systems in developing countries. Conversely, commercial competition for feed resources, especially fish meal and fish oil, and rising protest against environmental impacts by aqua feed usage and farm effluents are demanding less negative energy and nitrogen balances in nutrient conversion by cultured species and better environmental compatibility of aqua farms in developed countries. These tendencies are likely to reduce some of the differences in technological approaches and management strategies between developing and developed countries.

A greater technological homogeneity in the aquaculture industry is likely to facilitate technology transfers, farmer training and convergence of action towards the main obstacles that still lie along the path of an announced prodigious growth. The most crucial ones are the incidence of disease outbreaks, the effects of market price fluctuations or decline on enterprise operativeness and opposition by opinion groups and lobbies that are rightly concerned about environmental protection, conservation of biodiversity and animal welfare. It is generally recognized that research intensification and technological development can appropriately address these problems.

5. Fish Cytogenetics

Technical Advances in Fish Cytogenetics

The advance of fish cytogenetic research was greatly enhanced by technicial innovations in the preparation of metaphase chromosomes. The small size of fish chromosomes and their high number compared to mammalian chromosomes, and the then current "squash" or "crush" method of preparing chromosomes with direct fixation of tissue blocks in 50% acetic acid, often resulted in few high quality metaphase spreads for analysis. This "squash" method with acetic acid fixation generally also resulted in injury to the fingers. The chromosome preparation spreads for cytogenetic studies by using cells in suspension was a methodological breakthrough. After modifications, such as colchicine treatment, and optimization in hypotonization time for tropical marine and freshwater fish species, this method became common practice and is still used today. The more recent application of procedures for mitotic stimulation adapted for fish resulted in a significant increase in the number of available mitotic cells for the preparation of metaphase chromosome spreads. This paved the way for the consolidation of the protocol currently used with great success in most laboratories.

The growth of the fish geneticists' community not only multiplied the challenges for integration of the field, but also increased the experimental opportunities and approaches to solve problems in Neotropical fish genetics. In the 1980s, with three main centers for fish cytogenetics work, one located in Botucatu at São Paulo State University (UNESP), one in São Carlos at the São Carlos Federal University (UFSCar), and one in São Paulo at the

Biosciences Institute of the University of Sao Paulo (IB/USP), interest grew in Neotropical fish cytogenetics and went through a period of rapid expansion.

Cytogenetics Revealing Diversity in Fish

The first phase of work on fish chromosomes dealt mainly with gross comparisons between species. Owing to the techniques available, most publications described karyotypes and merely confirmed the diploid number of chromosomes in a given fish species, but might also include Giemsa staining of chromosomes, identification of heterochromatin (C-bands), the localization of nucleolar organizing regions and descriptions of a few meiotic metaphases. A positive aspect of this initial period was the observation of the great diversity in karyotypes of Neotropical fish species, with numerical and structural polymorphism, supernumerary chromosomes, sex chromosome heteromorphisms, *etc*. The remarkable diversity revealed in fish by application of cytogenetic techniques attracted the attention of taxonomists. Moreover, differences in diploid number and formulae seen in fish karyotypes raised intriguing questions: Do individuals of similar morphology but with different chromosomal numbers captured in distant hydrographic basins belong to the same species? Could each cytotype represent a different species?

The methodology used in fish cytogenetic studies passed through many modifications, broadening and diversifying the field of study. As a consequence, the field of fish cytogenetics, which had previously been only descriptive and confirmatory of karyotypic data, now began to investigate the structure and composition of chromosomes in detail. This was achieved by the use of base-specific fluorochromes, base analogues and restriction enzymes, which permitted the identification and localization of specific chromosomal segments and genes. More recently the application of fluorescent *in situ* hybridization (FISH) of cloned DNA probes to interphase and metaphase chromosome spreads was another major step that spurred fish cytogenetics research beyond the descriptive phase of karyotypes. The fusion of structural and molecular cytogenetics offers great potential for understanding the structure and composition of fish genetic material, and because these investigations are still in their infancy, there is much to be explored.

An Outlook

In the search for new areas and methods it is worth mentioning a few that might become the most promising. These include a better exploitation of the great diversity of fish species, the characterization of new species of interest for fish culture, the production of polyploid and gynogenetic fish, the induction of monosex individuals by hormone treatment, and the development of transgenic fish. While these new technologies offer novel and intriguing possibilities for further research and development of aquaculture stocks, their widespread application in fish genetics programs still faces diverse impediments, including some of legal, philosophical, religious and ethical nature.

With the development of techniques for high quality chromosome preparations and the recent application of fluorescent *in situ* hybridizations, the main focus in fish cytogenetics turns to defining chromosome structure and genomic mapping of fish. With the integration of molecular genetics and structural cytogenetics, many productive avenues of fish cytogenetic research are waiting to be fully explored that include: understanding of chromosome structure and evolution, chromosome polymorphisms linked or not to sex determination, the structure

and genetic functioning of sex chromosomes and supernumerary chromosomes, the latter feature commonly observed in fish karyotypes.

With the application of modern molecular genetics techniques, advances in our knowledge and conservation of the biodiversity of fish by the collaboration between cytogeneticists and taxonomists can be also anticipated. Multiple and multivariate analysis of molecular genetic marker studies and morphological data will allow for new interpretations of phylogenetic processes. This may well lead to a better or more complete understanding of the processes of speciation in fishes.

It is also important to reflect on the contributions made by icthyocytogeneticists over the years, not only in terms of scientific and technological advances, but also for the enhancement of the quality of life. When one considers the vast faunal diversity and genetic variability in fish, basic genetic research has broadened in a decisive way our knowledge of fish species and populations. This knowledge will lead in the short to medium term to the characterization of essential genetic banks and the establishment of necessary management and conservation programs for this fauna.

In the field of aquaculture, the application of new technologies along with appropriate management strategies has the potential to significantly increase food production. Already we are seeing a quantitative increase in the production of cultured fish using traditional selection methods for reducing culture time (*e.g.,* pacu), and the production of hybrids, sterile and monosex strains (*e.g.,* tilápias). The application of classic breeding, selection and hybridization methods for bloodstock improvement still plays a central role in the growing aquaculture industry, but novel genetic tools will soon complement these approaches. For example, chromosome and molecular markers have been developed and used for the identification of wild and cultured fish stocks, and it is now possible to ensure the maintenance of genetic variability in cultured stocks using these genetic tools. Finally, with the manipulation of fish chromosomes and perhaps transgenics, research conducted in laboratories and expanded to fish farms, will become an ever more important tool in the production and use of this of animal protein source as food.

6. Aquatic biotechnology

The realization of the role of aquatic biotechnology means that aquaculture in the coming decades should undergo a radical transformation driven by both engineering innovations (e.g., offshore submersible cages for mariculture monitored by remotely operated, camera-equipped underwater vehicles; inland water-recirculating systems for fish fattening' coupled to energetically complementary cultures, such as hydroponics, macroalgae or sponge cultivations; on-line process automation etc.) and bioengineering of aquatic organisms. In this scenario, the aquacultural products of the future will be raised with innovative methods and will appear somehow different from the products offered today to the consumers. Probably this vision may appear controversial, if not hazardous, not only to those that feel oppressed by technological progress and viscerally oppose biotechnologyMass media often depict biotechnology with a scaring profile or attributes unrealistic powers to it, so that it is mostly perceived, as an unnecessarily manipulative and intrusive option.

Actually, the development of biotechnological aquaculture is not a postponable option but rather an indispensable course to be taken if the projected feats of aquaculture must be

something more than wishful thinking. The objection that the incorporation of biotechnology into aquaculture is likely to exacerbate opposition by the anti-aquaculture lobby and further confuse the layman's opinion can indeed is reversed, if one considers that biotechnology preliminarily requires a stringent validation of its technical approaches in terms of environmental safety and biological harmlessness. Thus, it may actually provide solutions to still open problems about ecosystemic impacts by aquaculture. For instance, the enforcement of gonadal sterility in genetically manipulated fish to segregate them reproductively from wild conspecifics could be adopted as a general measure to avoid any genetic meddling between farmed escapees and wild fish. The search for viable alternatives to offset market saturation other than the recourse to new species for cultivation may also be pursued through hybridization, sperm cryopreservation and chromosome set manipulation. Molecular biology techniques provide faster and cheaper diagnostic assays for aquatic pathogens and recombinant DNA technology may be adopted to confer immunocompetence and is ease resistance (recombinant vaccines, antisense transgenes, DNA immunization, etc.). Inclusion of ethical issues into the guidelines for sustainable aquaculture is a sensible step to defuse the mounting impatience against the possible adverse consequences of aquaculture expansion. As to the technological upgrading to reduce sensitive interfaces and increase efficiency, the best strategy is to accelerate progress along multiple lines. These should include better farm design and management with appropriate measures for disease prevention and containment, the genetic improvement of broodstock according to the principles of quantitative genetics (selective breeding and crossbreeding of inbred lines), as well as productive improvements by chromosome and genetic engineering. There is only a short lapse of time ahead of us to solve the present emergencies and endow the aquaculture sector with the means and technologies to attain its goals. All opportunities must be explored and compared without preconceptual exclusions. In this paper, an effort is made to outline the main advantages and problems associated with the application of the techniques of chromosome set manipulation, sex control, intergeneric hybridization and gene transfer in fish culture, with particular reference to cultured Mediterranean marine teleost fish. The complementarity and integrability of these techniques will be briefly discussed.

6.1 Chromosome set manipulation

In fish culture, the techniques of chromosome set manipulation can be used for two different purposes: either to induce polyploidy, essentially to obtain triploid and tetraploid fish, or to reproduce fish by uniparental chromosome inheritance to obtain gynogenetic and androgenetic fish. The main aspects of this "chromosome engineering" have been reviewed. Two points are worth considering: firstly, changes from diploidy to polyploidy and uniparental reproduction may occur naturally in teleosts. For instance, salmonids have apparently undergone a tetraploidization event to their ancestry and spontaneous triploids and tetraploids are sometimes found within diploid fish populations. A round or two of tetraploid evolution is surmised even in the piscine ancestors of mammals. Gynogenesis is the sole mode of reproduction of some populations of the crucian carp *(Carassius auratus gibelio)* and several species of the family Poecilidae, while spontaneous gynogenesis or androgenesis is sometimes observed in crosses of different fish species. Secondly, the techniques of chromosome set manipulation can be usefully applied to complement other techniques, such as interspecific hybridization, artificial speciation, genetic engineering, sex control, population control, selective breeding, pure line or clone crossbreeding and sperm cryopreservation.

6.2 Induction of polyploidy

The main procedures for the induction of polyploidy in fish consist in either the blockage of the resumption of the second meiotic division in the fertilized oocyte with consequent retention *of* the second polar body (meiotic block) or the suppression of the mitotic division at the first embryonic cleavage with consequent fusion of the two blastomere genomes (mitotic block). Because at syngamy, during normal fertilization, a diploid secondary oocyte is fused with a haploid spermatozoon, the retention of the haploid chromosome set of the second polar body induced by the meiotic block will result, at karyogamy, in a triploid chromosome complement. Conversely, the doubling of the diploid chromosome seot f the zygote caused by the mitotic block will result in a tetraploid status. Several methods can be utilized to induce meiotic or mitotic blocks, such as a thermal shock (heat shock at a sub-lethal temperature or cold shock at $0-4^0C$), a hydrostatic pressure shock, electrofusion (electric field-induced cell fusion) or exposure to chemical fusogens (e.g., nitrous oxide, cytochalasin B, polyethylene glycol). All these treatments must be applied just before the presumptive time of meiotic resumption or mitotic division. Thermal and pressure shocks are usually preferred to the other methods because they are potentially less traumaotric toxic. Ploidy levels can be assessed by a variety of techniques, including measurement of erythrocyte nuclear or cellular volumes, nucleolar counting, chromosome counting, and DNA content determination by flow cytometry.

Triploidy

Interest in triploid fish arises from the fact that they are mostly sterile owing to either a lack of gonadal development beyond a rudimentary differentiation stage with consequent gametogenetic failure (gonadal sterility), or lack of gamete fertility due to meiotic abnormalities caused by the odd number of chromosome sets (gametic sterility). The severity of gonadal development inhibition varies with species and sex. Generally, triploidy inhibits more ovarian than testicular development, because it does not interfere with the multiple waves of spermatogonial mitotic divisions. Moreover, secondary sexual characters and sexual behaviour are not always suppressed in males.

There are a number of possible benefits associated with gonadal sterility, such as:

(i) In relation to growth and survival: (a) faster growth rate of triploids after the normal time of puberty of diploids, as the former do not shunt energy from somatic growth into gonadal maturation; (b) increased longevity and growth to trophy size in released fish that normally experience high mortality at the time of spawning, like many salmonids; (c) lack of the decrement in the bactericidal activity of serum observed in diploid fish during gonadal maturation and spawning; and (d) intensification of flesh colour in salmonids fed canthaxanthin at the end of the fattening stage, when this step is performed during the normal breeding season.

(ii) In relation to population control: (a) population controinl case of prolific species which tend to overpopulate confined water bodies; and (b) population control in case of introduced allochthonous species.

(iii) In relation to hybridization: (a) enhanced survival of certain interspecific and intergeneric hybrids, whenever genome incompatibility is mitigated by the double maternal gene dose; (b) prevention of introgressive hybridization, when a restocked variety or species can interbreed with a threatened sympatric variety or species; (c) avoidance of backcrossing between partially fertile interspecific hybrids and their parental species; and (d) reproductive segregation of fertile interspecific hybrids.

(iv) In relation to transgenesis: reproductive segregation of genetically engineered fish from wild conspecifics.

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The main drawbacks in the exploitation of induced-triploidy are: (i) difficulty in obtaining 100% triploid fish by meiotic block due to rejection of the sperm genome or failure of second polar body retention; (ii) lower prepubertal growth rate of triploids in some species, especially when triploids are cultured communally with diploids; and (iii) lower respiratory capacity of triploids due to a less favourable surface to volume ratio in red blood cells and, at least in rainbow trout, *Oncorhynchus mykiss,* a lower blood haemoglobin concentration leading to macrocytic anaemia and dimished survival and growth at chronic high temperatures.

Tetraploidy

Tetraploidy has been less intensively investigated than triploidy and its potential advantages are still to be fully explored. Newly arisen tetraploids are often less viable than diploids and triploids and fertility may be reduced in autotetraploids because of unbalanced disjunction of chromosomes after meiotic multivalent pairing. Theoretically, induced tetraploid fish may be exploited:

(i) In relation to genetics: to produce super heterozygous fish with up to four different alleles per locus and increased enzyme multiplicity.

(ii) In relation to two-step chromosome set manipulation: (a) to generate autotriploid progenies with a lower level of homozygosity when tetraploid fish are crossed with isospecific diploids; and (b) to obtain androgenotes by fertilizing genetically inactivated eggs with diploid sperm from autotetraploid males.

(iii) In relation to hybridization: (a) to create new tetraploid hybrid species by polyploidization of interspecific hybrids into fertile allotetraploids (artificial speciation); (b) to produce sterile allotriploids when an autotetraploid is crossed with a diploid mate of a different, genetically compatible species, or alternatively when an allotetraploid is crossed with a diploid mate of one of its original parental species; (c) to obtain sterile triple-hybrid allotriploids by crossing an allotetraploid with a diploid mate of a species different from the parental species of the allotetraploid.

Although polyploidization is recognized as a basic mechanism for fast speciation in nature and a dynamic force in evolution, it should be noted that bringing into existence a new hybrid species by induced allotetraploidy entails the risk of ecosystemic impacts on wild fish populations and other aquatic organisms, especially when the hybrids possess great invasiveness and good fertility. Regulations about their containment must, therefore, be assimilated to those for either allochthonous species (as synthetic species are foreign to the local fauna) or genetically modified organisms (as two phylogenetically distinct sets of genes are recombined into a novel perpetuating germline). Uniparental chromosome inheritance Fertilization using either eggs or sperm whose genetic material has been inactivated by radiation gives rise to haploid gynogenetic and androgenetic fish, respectively. Ionizing radiation (y-rays and X-rays from 60cobalt and radium sources; dose range: 30-150 krad) shatters DNA, whereas short-wave ultraviolet light (UVc; wavelength: about 254 nm; dose range: 3-40 kg mm) destroys DNA by thymine dimerization. Though less penetrating, UVc light is preferred to ionizing radiation because: (i) it does not require hazardous machinery unavailable at fish farms; (ii) it does not leave supernumerary chromosomal fragments, residual from the irradiated gamete, inside the zygote nucleus, where they can be transcriptionally active and replicated during embryogenesis; and (iii) it results in better morphology of surviving haploid embryos or larvae. Ionization radiation is, however, needed to treat opaque yolky oocytes or large volumeosf undiluted milt.

In teleosts, haploidy is usually incompatible with survival beyond embryonic or early larval stages, though a low viability with heavy abnormalities has been observed in haploid tilapia. Diploidization of the maternal chromosome complement in gynogenotes can be carried out by either meiotic block (meiogynogenesis) or mitotic block (mitogynogenesis), using the same techniques adopted for polyploidization. Androgenotes, instead, can be diploidized exclusively by mitotic block. To confirm uniparental chromosome inheritance, special control measures can be taken in the selection of the parent whose gametes are genetically inactivated. This may be characterized by a visible dominant trait which is absent in the other mate and shows up in biparental progeny. Alternatively, it may belong to a different species whose hybrids are phenotypically divergent or unviable in the diploid and triploid conditions.

Meiogynogenesis

Meiogynogenotes are in general only partially homozygous, their heterozygosity level depending upon the frequency of gene recombinations caused by odd numbers of crossovers between homologous chromatids. Despite their lack of complete homozygosity, meiogynogenetic fish can be utilized for a variety of purposes, such as: (i) In relation to triploidy induction: to define the best conditions for triploidy induction as indicated by the best survival of gynogenetic progeny after meiotic block. (ii) In relation to sex control: (a) to explore the genetic sex determination mechanism and the influence of environmental factors, if any, on the final sexual phenotype; (b) to produce directly allfemale progenies in species with genetically determined sex, female homogamety and better performancein culture of females than males; (c) to overcome the difficulty in the discrimination of pseudomales (genetic females inverted into phenotypic males) from genetic males, whenever meiogynogenotes are all-females, thus yielding, after sex reversal, all pseudomales; the latter can be crossed to normal females to generate all-female progenies (monosexualization of fish stocks). (iii) In relation to genetics: (a) to discharge part of the genetic load of harmful recessive alleles from a founder broodstock gene pool, because offspring homozygous for such alleles would be phenodeviant and defective and, thus, discarded; (b) to facilitate mapping of genes relatively to their centromeres, especially in species with many chromosomes, because the closer a locus to its centromere, the lower the frequency of heterozygotes in the meiogynogenetic progeny; and (c) to estimate the degree of conservation of gene arrangements and differences in gene recombination rates among related species.

Mitogynogenesis

Mitogynogenotes are completely homozygous, because their genome derives from a mitotically duplicated haploid chromosome set. Their viability, sex and fertility have been explored in experimental fish models, such as the zebrafish, *Danio rerio* and the medaka, *Oryzias latipes*, as well as in rainbow trout. Their potential use in fish culture has been examined in the common carp, *Cyprinus Carpio*, flounder, *Paralychthys olivaceous*, ayu, *Plecoglossus altivelis*, gilthead seabream, *Sparus aurata,* and European seabass, *Dicentrarchus labrax*.

Several possible applications of mitogynogenesis can be envisaged, such as:
(i)   in relation to genetics: (a) to free completely a broodstock gene pool from its load of deleterious recessive alleles, because all of them will be manifested in the offspring and, thus, culled; (b) to assess phenotypic variation due to environmental factors in the estimate of genetic heritability (h2) for quantitative traits, as clonal fish have no genetic

variation; and (c) to study the influence of homozygosity on developmental stability, as measured by fluctuating bilateral asymmetry for meristic traits (fin rays, gillrakers on the first branchial arches, mandibular pores etc.).

(ii)   In relation to cloning: (a) to produce fish clones whenever F1 mitogynogenotes which are homozygous but not mutually isogenic due to maternal allele recombinations by meiotic crossovers, are reproduced gynogenetically yielding, at FP, an isogenic homozygous offspring (autozygous clone) from each mitogynogenetic clonal progenitor; (b) crossing of normal and sex-inverted fish from two different clones to obtain, at F3, heterotic interclonal hybrids (allozygous clone) with enhanced performance in culture ; (c) crossing of several clones to form a multiclonal broodstock, which can be subjected to intense selective pressure withoaunty risk of inbreeding depression.

(iii)   In relation to histocompatibility and disease resistance: (a) to analyse the histocompatibility gene system in fish by determining the fate of skin allografts exchanged among different clones; and (b) to discriminate innate disease resistance by challenging a set of clones with different pathogens; certain clones of common carp exhibit a genetically predetermined low antibody response and high susceptibility to infection.

(iv)   In relation to transgenesis: to achieve homozygosity of transgenes in the mitogynogenetic progeny of clonal, or even non-clonal, transgenic fish with an initial hemizygous or mosaic transgene distribution to set up stabilized transgenic fish lines with 100% transmission of the transgenic trait.

It should be noted that, in the course of time, isogenicity may not be maintained in autozygous clones in the presence of an intense spontaneous mutation rate. In zebrafish, an isogenic clone became polyallelic at a malate dehydrogenase locus after 10 bisexual generations. So, recloning may be necessary to ensure genetic uniformity that may be checked by DNA fingerprinting.

Fragment gynogenesis

Fragment gynogenesis, consisting in the inclusion of paternal chromosome fragments, inherited from y-ray-treated sperm, in the gynogenome, has been attempted in rainbow trout. This approach would be equivalent to a chromosome-mediated gene transfer and may be exploited: (a) to map genes on chromosomes, especially in case of hybrid gynogenotes; (b) to aid in the identification of major genes for important traits, like sex determination; and (c) to create auto- or allotransgenic fish for multiple genes donated by iso- or allospecific males, respectively, whenever centromere-bearing fragments are diploidized by mitotic block and perpetuatedin the descendants.

Androgenesis

Similarly to mitogynogenesis, diploid androgenesis, which involves the genetic inactivation of the egg by irradiation with doubling of the fertilizing sperm chromosome set, is also a fast method for producing fish clones in only two generations. This is much faster than the production of pure lines by multiple sibmating.

Egg irradiation, however, may damage not only nuclear DNA but also mitochondrial DNA and ooplasmic RNAs which are essential for embryonic development. Low survival of androgenotes has been reported in various species, with some improvement when diploid sperm from tetraploid males was used for androgenesis. Moreover, low fertility was observed

in both androgenetic males and females of rainbow trout and viability of YY individuals must be established in each species with XY sexual chromosomes.

Despite these drawbacks, androgenesis has two important applications:

(i) In relation to sex control: (a) to produce directly all-male progenies in species with genetically determined sex, male homogamety, and better performance in culture of males than females; analogously to meiogynogenotes, these androgenotes may then be sex-inverted into pseudofemales to produce all male stocks when crossed with normal males; and (b) to obtain YY males in case of male heterogamety (XY) that may be utilized to generate an all-male progeny when crossed with normal females (XX). (ii) In relation to sperm cryopreservation: to ensure recovery of fish clones or natural ecotypical strains from their cryopreserved sperm stored in gene banks for perpetuation or conservation genetics programs.

Furthermore, androgenesis may provide a tool for artificial speciation in several still theoretical ways, such as: (a) fertilization of genetically inactivated eggs with sperm from partially fertile diploid interspecific hybrids and subsequent diploidization by mitotic block; any viable fish could then be used to found new diploid hybrid species, each receiving variable genomic contributions from the original parental species; and (b) fertilization of genetically inactivated eggs with sperm from another species and subsequent diploidization to give rise to nuclear-cytoplasmic hybrids. Containment of these fish should be equivalent to that of allotetraploids outlined above.

6.3 Chromosome set manipulation in marine fish culture

Chromosome set manipulation has been investigated and applied to increase productivity mainly in salmonids, cyprinids, cichlids and ictalurids. Research on farmed marine fish species, mostly belonging to the families Pleuronectidae, Moronidae and Sparidae, has generally begun more recently. Meiotic and mitotic blocks have been carried out with good yields (mostly >75% and up to 100%) in both seabass and seabream using cold shocking (0-2°C for 13-20 min at 3-5 min post-fertilization, PF) and pressure shocking (58.8-88.2 MPa for 37 min at 40-90 min PF), respectively. Meiotic block is used to produce triploids and meiogynogenotes, while mitotic block gave rise to tetraploids and mitogynogenotes. As shown in Figure. 1, the ploidy level of haploid, normal diploid, triploid and tetraploid seabream was evidenced by their respective karyotypes and was correlated with the highest number of nucleoli per nucleus in embryonic or larval cells. Analogously, Fig. 2 shows the karyograms of similarly manipulated seabass.

The efficacy of cold shocking in the mass production of triploid seabass was also confirmed. Conversely, heat shocking was found to be poorly effective (25% yield) at 29°C for 25 min at 15 min PF but very effective (83% yield) at 32°C for 10 min at 13 min PF, although 59% tetraploids were also concomitantly produced. Low percentages of triploidy in seabass were observed with pressure shock (58.8 MPa for 2-3 min at 5 min PF) and cytochalasin B treatment (0.5-1 mg/l at 5 min PF). Among other species of the family Moronidae, triploidy was induced in striped bass, *Morone saxatilis*, and hybrid meiogynogenotes were obtained from eggs of sunshine bass (female *Morone chrysops* X male *M. saxatilis)* fertilized with inactivated sperm from white perch *(Morone americana)*.

In *S. aurata,* 100% triploidy was reportedly induced by heat shocking (34°C for 10 min at fertilization). Heat shocking at a rather high temperature (36.5-37.3"C) was also

successfully applied to gilthead seabream eggs fertilized with normal and UV-inactivated sperm of Japanese red seabream, *Pagrus major,* giving rise to meiogynogenetic and triploid hybrid offspring. Retention of the second polar body was accomplished in red seabream obtaining triploids by cold shocking and meiogynogenotes by both cold and heat shockings.

Androgenesis has not been attempted so far in *D. labrax* and *S. aurata* despite the fact that the transparency of ovulated eggs in these species may allow their genetic inactivation by exposureto UV light instead of ionizing radiation.

To provide clues for future research, the following points and new results are worth considering:

(i) Thermal shocks are ineffective in blocking the first mitotic division in both seabass and seabream, as also found in red seabream.

(ii) Tetraploids and mitogynogenotes of seabass and seabream show lower survival at hatching and during subsequent development as compared to triploids and meiogynogenotes. In particular, when a lot of seabass, subjected to mitotic block and containing 56% tetraploids and the rest diploids at hatching, was raised for ten months, it was found that tetraploids were reduced to 33% after one month and was no longer present at eight months, leaving only surviving diploids. Therefore, purely tetraploid seabass should be grown to establish whether they are outcompeted by diploids or merely short-lived.
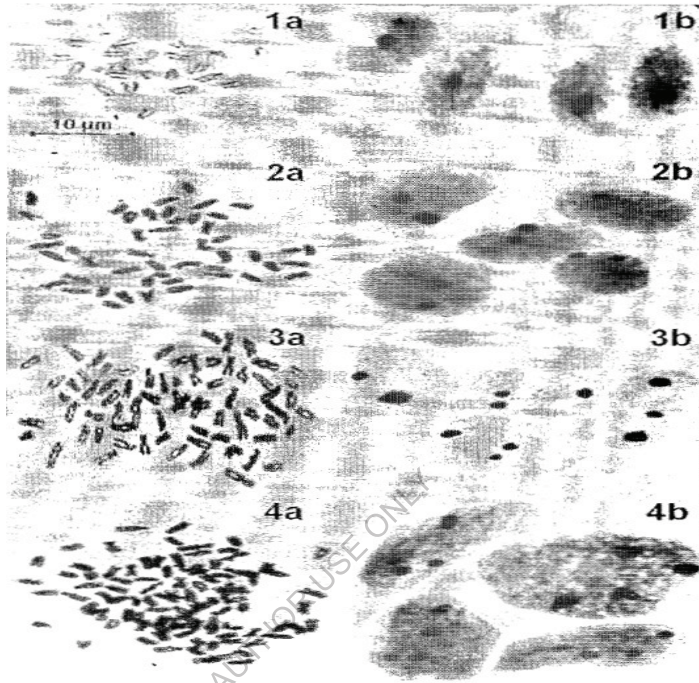
Figure 1. Metaphase chromosome plates (la-4a) and nuclei of embryonic or larval cells with nucleoli evidenced by silver nitrate staining (1 b-4b) obtained from gilthead seabream *(Sparus aurata)* haploid (la,b), normal diploid (2a,b), triploid (3a,b) and tetraploid (4a,b) individuals.

(iii) Triploid seabass appear to be sterile. In triploid females, the weight of the ovary at 2 years of age was only 14% of that of control diploids. The ovigerous lamellae contained mainly oogonial nests with only few scattered primary previtellogenic oocytes, indicating that initiation of meiosis was impaired. In triploid males, the weighto f the testis was significantly lower than that of diploids at 3 but not at 2 years of age. In controls, the testicular lobules were full with cysts at all stages of spermatogenesis, while in triploids germ cells were represented only by scattered spermatogonia and more abundant primary spermatocytes. Thus, triploidy in seabass hinders the onset of meiosis but not gonial proliferation in both sexes, as reported also in red seabream.
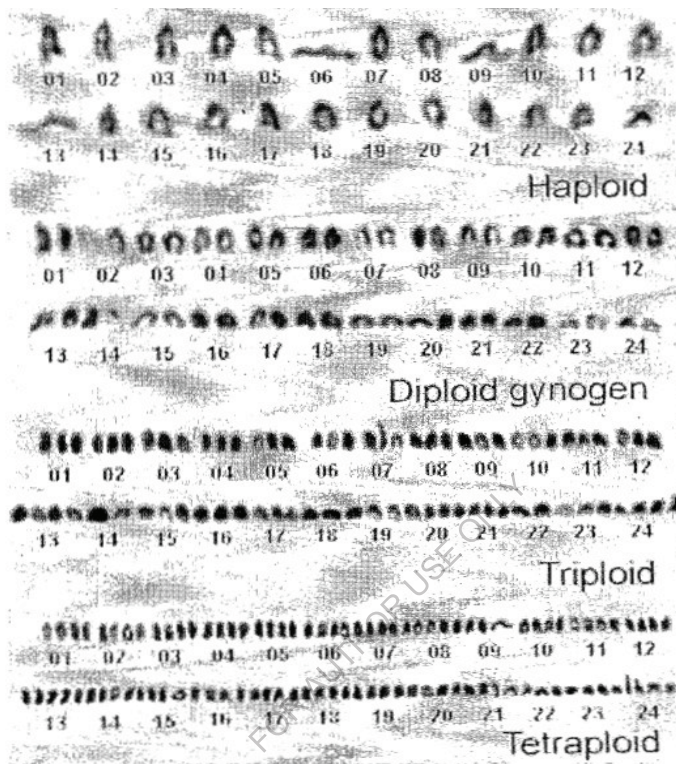
Figure 2. Karyograms derived from metaphase chromosome plates obtained from European seabass *(Dicentrarchus labra)* haploid, meiogynogenetic diploid, triploid and tetraploid individuals.

(iv) Throughout the prepubertal period, the growth rate of triploid seabass remained significantly lower than thaot f diploids in two distinct trials. In the first one, the mean body weight a2t *0* months was 73% of that of normal diploids (115 *vs.* 158 g, n = 11O; P<0.05); in the second one, at 31 months, it was 66% of that of meiogynogenetic diploids (241 *vs.* 365 g, n = 72 and 36, respectively; P<0.05). At 3 yr of age, however, the divergence between triploids and diploids became no longer significantly different (1129 *vs.* 1241 g). This suggests that, because sexual maturity is dependent upon size and is usually attained at the enodf the 2nd year in males and of the 3rd year in females, the culture of triploid seabass would be convenient just to produce fish of big market size or suitable for filleting. In red sea bream, no difference in growth rate was observed between diploid and triploid full-sibs.

(v) During on growing for 16 months, the mean body weight of meiogynogenetic seabass was comparable to that of normal diploids but was significantly lower at 20 months

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of age (126 vs. 158 g, n = 25 and 110, respectively; P<0.05), though still greater than that of triploids. Actually, the difference merely in the last weight values may be fortuitous and cannot be regarded as a conclusive proof of a lower performance by meiogynogenotes. In seabream, no significant difference was observed in growth rate between meyogynogenetic and normal diploids up to 6 months of age.

(vi) The possibility of spontaneous diploidization in a small percentage of untreated gynogenotes by retention of the second polar body has been verified in both seabass and seabream. A similar phenomenon has been described also in the common carp.

(vii) Despite the fact that seabass meiogyno denotes bear only maternal chromosomes, they can spontaneously differentiate into males. This observation, coupled to the fact that cultured diploid seabass often display sex ratios strongly skewed in favour of males (3:l), but sometimes also in favour of females, hint at a more complicated mechanism of sex determination in this species than earlier suspected. As summarized in Table 1, in four different experiments at three locations, the sex ratio of the control diploids ranged from4: l in favour of females at Pellestrina to 351 in favour of males at Eilat, but included also a sex ratio of 1:l at Monfalcone. These data seem to point at a thermal influence on sex determination because fingerlings at the hatchery of Pellestrina are raised at a lower water temperature than that at Eilat, while Monfalcone stayins between as it utilizes the effluents of a power station. Significantlyi,n the Nile tilapia, *Orechromis niloticus,* exposure of fry to high temperature was found to increase the proportion of males. However, the observed sex ratios of the meiogynogenotes do not conform to the assumption that males prevail at higher water temperatures. As a matter of fact, while meiogynogenotes differentiated more into females, the relative abundance of males was greater at Pellestrina than Eilat, and thus it was inversely related to the water temperature. It is really surprising that, at Pellestrina, there were fewer females among the meiogynogenotes than in control diploids. Recently, in a lot of seabass meiogynogenotes raised at a fish farm in Sicily, 58% were found to be males (P. Benedetti, personal communication).

A differentiation of gynogenotes into males has been reported also in the Nile tilapia. In this species, a dual system of sex chromosomes with homogametic and heterogametic individuals of the same gender has been proposed. In another teleost, *Leporinus elongatus,* the sexual heterochromosomes may recombine yielding atypical sex karyotypes and unbalanced sex ratios. Clearly, more data are necessary to disentangle genetic from environmental effects and, at present, the mechanism of sex determination of seabass remains obscure. An interesting experiment would be to establish the sex ratio in F1 meiogynogenotes derived from female meiogynogenotes (or even better mitogynogenotes) and raised under different environmental conditions.

(viii) Complete sexual inversion of either sex by precocious administration of heterologous sex hormones with the diet can be easily accomplished in seabass, similarly to other teleosts.

Feeding 17a-methyltestosterone at the dose of 10 mg-kg' food during 100 or 200 days starting 126-226 days post-hatching increased the percentage of males from 79% in the controls to 100%. Treatments started after 226 days post-hatching were ineffective. The same androgen dose given earlier (65 and 95 day-old fish) and for shorter periods (75 and 100 days) also resulted in 100% males. Further,' masculinization was equally effective in 90-day-old fish at same dose applied for only 30 days or at a IO-fold lower dose (1 mg androgen-kg-' food) for 60 days. At 23 months of age, pseudomales were fluent with milt, indicating a

functional sexual inversion. Given the great sensitivity of seabass to androgen, treatment may be probably attenuated even more. This species is also responsive to estrogen administration because a high dose of estradiol (125 mg-kg' food) given to 65- and 95-day-old fish for 75-106 days yielded 100% females. In this case too, it is worth exploring lower dosages and shorter periods of treatment.

All-female stocks of seabass would be certainly more productive in culture, as females grow faster than males and mature sexually 1-2 years later. Nonetheless, if, unlike in trout farming, all-female progenies cannot be obtained by crossing normal females with pseudomales due to the problems discussed at point 7, then direct feminization might be an alternative to be considered. In the European Community, this would require the addition of a specific authorization in the existing Directive.

This forbids the administration to aquaculture animals of substances with androgenic or estrogenic action but authorizes the treatment of young fish for the first 3 months with allowed androgen under veterinarian control for the purpose of sex inversion. The extension of this exception to natural estrogens to feminize seabass appears not to be objectionable on a scientific basis under specific circumstances, such as: (a) Inclusion in the feed of fish ovarian meal, like ground dried trout ovaries which are a waste product of the filleting process but are enriched in estrogen at maturity; alternatively, preparation of an equivalent feed obtained by the direct addition of estradiol-17ß in a similarly low concentration; and (b) Administration of such feeds to seabass just after weaning and for less than three months, especially if raised in a closed system with recirculating water; otherwise, the water effluent may undergo a depuration step (biological filtration, lagooning, etc.) to cut down any residual estrogen leakage into the environment. This approach would be absolutely safe for the consumer because no measurable trace whatsoever of exogenous estrogen would be left in the meat after more than one year in culture. It is noteworthy that estrogen production in fish is more widespread than in mammals, as substantial quantities are produced also by the brain and other organs, besides the ovary. This normal endogenous concentration would overshadow any improbable remnant of feminizing estrogen.

(ix) Hormonal sterilization has not yet been carried out in seabass, but partial sterilization was reported when masculinizing or feminizing treatments were performed in older fish, i.e., at 115 and 145 days of age.

Complete sterilization is likely to require prolonged hormonal exposure, as in other species, but this engenders problems about consumer safety and acceptance, environmental protection against hormonal residues, and producer's convenience because seabass growth was found to be adversely affected by feeding androgen for more than 100 days. Therefore, at present, this approach seems impractical.

6.4 lnterspecific and intergeneric hybridization

lnterspecific and intergeneric hybridization has contributed greatly to genetic and productive improvements in agriculture but much less so in terrestrial animal husbandry, which has mostly privileged interracial (intraspecific) hybridization. In fish culture, however, interbreeding of different species or genera may be of greater practical value than generally believed, especially in marine fish farming. Facilitated by their prevalently external mode of reproduction, high fecundity, frequent mass spawning habits, and rather common reciprocal genetic compatibility, teleosts can hybridize spontaneously in nature and are relatively easy

to hybridize in culture. Actually, hybrids have been produced within each bony fish family that includes species breeding in captivity.

Natural hybridization

Closely related species with isochronous reproductive seasons, and especially those relying on communal spawning rather than on complex courtship patterns, are more likely to hybridize in nature. Spontaneous hybrids, however, are relatively rare among marine species as compared to those living in freshwater. Here, environmental instability precipitating confinement of different species within a restricted water body, reduction of spawning grounds by man-induced habitat alterations, massive restocking with kindred species or introductions of alien species are often cited as causes of hybridization in fishes. The lesser importance of these factors in the marine environment and its relative stability may explain the rarity of marine fish hybrids.

Among freshwater teleosts, intergeneric hybrids are common in the family Cyprinidae, whereas hybrids tend to be intrageneric in the other families. Normally, only FI hybrids of wild spawners are found in nature, sometimes as reciprocal hybrids. Introgressive hybridization by backcrossing and multiple hybridization occur exceptionally because of hybrid sterility, reduced fertility or inadequate mating ability. They are nevertheless reported, as the finding of a natural tripleb ass hybrid resulting from the crossing between a hybrid (probably *M. chrysops* X *M. saxatilis)* and a third species, the yellow bass, *Morone mississipiensis.* Sometimes, as in salmonids, F2 hybrids are less fit than F1 hybrids.

Artificial hybridization

In culture, straight F1 hybridization can be carried out by: (i) volitional tank spawning of captive females of one species held in cohabitation with males of another species, particularly if gamete release is stimulated by exogenous hormones; (ii) artificial fertilization of the eggs of one species with the fresh sperm from a different reproductively synchronous species, or vice-versa to generate the reciprocal hybrids; and (iii) artificial fertilization of the eggs of one species with the cryopreserved sperm from another species, thus multiplying the number of possible crosses by inclusion of males belonging to nonsynchronous or exotic species. Whenever F1 hybrids are at least partially fertile, there are five conceivable options for further hybridization, such as: (i) breeding of equivalent hybrids from the same parental species (F2 hybridization); (i) intercrossing of reciprocal hybrids from the same parental species (F2 reciprocal hybridization); (iii) intercrossing of hybrids from different parental species, with four possible combinations of hybrids and their reciprocal hybrids (double hybridization); (¡v) backcrossing of hybrids with one or both of their parental species (introgressive hybridization); and (v) crossing of hybrids with a third non-parental species (rehybridization).

Hybridization and selection

In all these cases, owing to the casual segregation of the parental chromosomes during gametogenesis in the F1 hybrids, the genetic contributions of each original parental species (F0) to the diploid chromosome set of each new F2 hybrid will be totally at random. This genotypic heterogeneity among F2 hybrids is likely to be associated with phenotypic heterogeneity in terms of performance in culture. Apart from introgressive hybridization that, through successive crosses, tends to disperse the initial shuffling of heterospecific

chromosomes within the broader genetic pool of the parental species, thus minimizing genotypic heterogeneity, the latter will be perpetuated when applying F,, hybridization or augmented with double hybridization or rehybridization. The picture may be further complicated by crossovers between parental chromosomes, if present. This means that selection of F, hybrids for complex traits will be much more difficult to control than in pure species because of the excess of variation, unless backcrossing is introduced at an early stage. The introgressive approach was successfully adopted in the Soviet Union where the intergeneric hybrid of the common carp X crucian carp, *Carassius carassius,* was backcrossed to confer upon one parental species distinctive traits of the other parental species. Hybrids between *Oncorhynchus mykiss* and brown trout produced some trout of golden appearance; but the hybrid, as expected, did not breed true.

Reports of this sort are sporadic and scarcely documented but they are of interest being examples of (multiple) gene transfer by conventional breeding techniques. The fact that foreign genes were inserted into a species by genetic intermingling through hybridization rather than by genetic addition through egg microinjection or gamete electroporation does not seemto justify, in such trials with fertile fish, the total lack of those containment measures that are usually imposed on experiments with transgenic fish.

The use of interspecific hybridization as a preliminary step to selective breeding appears to be of questionable practical advantage because of the frequent low fertility of hybrids, the long period of time required for the introgression of the heterospecific trait by repeated backcrossing, the risk of the accidental dispersal of the fertile hybrids or their introgressed descendants into the natural environment during the handling of the numerous fish under selection, and the possibility of losing the heterospecific trait during introgression. Rather than transferring genes as a result of hybridization followed by selective introgression, it looks much more convenient to postpone hybridization to gene transfer by gamete or embryo manipulation to exploit hybrid sterility for the reproductive containment of the transgenic fish, as suggested below.

Hybridization and chromosome set manipulation

Another attractive approach is the integration of interspecific and intergeneric hybridization with chromosome set manipulation techniques, as outlined above. Allotriploid fish obtained either by hybridizing two diploid spawners belonging to different species with subsequent retention of the second polar body, or by crossing allotetraploids with diploid mates, should be absolutely sterile because of both their hybrid nature and triploid condition. Allotriploids produced by crossing female *S.aurata* X male *P.* major, with second polar body retention, could be differentiated phenotypically from diploid hybrids, even at the fingerling stage, thus making a check on ploidy status directly feasible (Gorshkov*et al.,* 1995). Induction of triploidy may be applied to hybrids from taxonomically related species to eliminate any residual fertility and risk of introgression in nature with the parental species. This risk is often neglected in fish culture on the grounds that, even if fertile, hybrids are not inclined to reproduce in the wild, as indicated above. Such an argument may be further strengthened whenever equivalent or similar hybrids arise sometimes spontaneously in nature due to weak interspecific boundaries.

Triploidy may alsob e the natural outcomeo f a hybridization event. Females of the reciprocal crosses *O. latipes* X Oryzias curvinotus show reduced fertility and ovulated diploid eggs with one complete chromosome set for each parental species so that, when backcrossed,

they give rise to a triploid progeny. A similar finding was reported also for the hybrids of the cross crucian carp, *C. auratus gibelio* X common carp.

In comparison with proximal hybrids, sterility is expected to be more severe or complete in diploid hybrids born from systematically distant species, like in intergeneric hybridization, owing to unsuccessful pairing of the homologous chromosomes. As a consequence, gonadal development is often markedly impaired. Partial genetic hybridization achieved by both fragment gynogenesis and nuclear cytoplasmic hybridization by either androgenesis or nuclear transplantation is of great experimental interest, but it poses containment problems for farming because this kind of artificial hybrids is not expected to be sterile. A technique for the production of nuclear cytoplasmic hybrids by transplanting nuclei from common carp eggs into the cytoplasm of *C.* auratus eggs. It was shown that the expression of meristic traits, such as the number of vertebrae or pharyngeal teeth, is influenced by both the nucleus and the cytoplasm.

Advantages of hybridization

Besides sterility, hybrids may display other positive traits that nevertheless cannot be predicted from the knowledge about their parental species and may be absent altogether, particularly in certain teleost families. Very few of the interspecific and intergeneric hybrids of salmonids and cyprinids have any farming or restocking potential because of their low viability or inferior performance with respect to the parental species. When things go otherwise, there are five possible outcomes of hybridization which may result beneficial: intermediacy, combinability, luxuriance, monosexuality and novelty.

Intermediacy, that is the fact that morphometric and meristic traits of the hybrids are somehow intermediate between those of the parental species, has been confirmed in several cases. This may create more appealing forms of fish, particularly in ornamental fish culture.

Combinability refers to the possibility of combining in the hybrids favourable characteristics proper of each parental species. For instance, the sturgeon hybrid, called the bester, combines the fast growth rate of the great beluga, *Huso huso*, with the freshwater tolerance of the tiny sterlet, *Acipenser ruthenus*. As the bester is a fertile, self-perpetuating breed, it provides an example of artificial speciation tolerated because of its benefits.

Luxuriance is an important goal of artificial hybridization and is mostly meant to denote a superior growth rate of the hybrids with respect to the parental species. Enhancement of at least early growth rate is a recurrent feature in several hybrids and may reflect the attenuation of inhibitory regulations on growth whenever the underlying molecular mechanisms inherited from the parental species result incompatible or poorly harmonizable within the hybrid. For instance, the reduction of the energy expenditure for defence metabolism, especially which involved in the reaction to stress stimuli, may divert more energy towards auxogenic processes. Interestingly, the sunshine bass (female *M, chrysops* X male *M.* saxafilis) shows a lower responsiveness to stressors than the paternal species, as measured by the increase in plasma cortisol level induced by a standardized confinement stress. This trait, that would be hazardous in nature because it reduces alertness towards threats, becomes a positive attribute in culture because it leaves more energy for a faster early growth rate and increases resistance to infection, and hence survival, as a consequence of lighter corticosteroid immune-suppression. Luxuriance should not be confused with heterosis, i.e., the improvement of performance that marks certain crosses' between conspecific inbred

lines. Luxuriance is the outcome of hybridization per se, that is the mixing of genomes belonging to distinct phylogenetic lines, and is likely to be more evident in wider taxonomic crosses, while being relatively independent from the particular genotypes of the spawners. The superiority of the luxuriant hybrids with respect to both parental species is more easily explainable as a subtractive effect, namely the loss of an unwanted function while retaining all other vital capacities and homeostatic power, hence a fortunate compromise, rather than as a synergistic complementarity between parental traits. This luxuriance event is more likely to occur when the parental species have genomes that are equivalent in size and chromosome number. Otherwise, aneuploidy and developmental abnormalities may ensue.

If F1 hybrids are fertile, luxuriance may not be maintained in F2 hybrids because of the aforementioned random recombination of the parental chromosomes, and because crossovers, if any, between parental chromosomes in the FI generation will cause chromosomal hybridization, i.e., exchange and incorporation of heterospecific chromosomal segments, with possible detrimental positional effects among genes linked within the same chromosomes. This would be in agreement with the observed inferior fitness of salmonid hybridism the F2 progeny than in the F1.

Conversely, heterosis is not due to interbreeding by itself but depends upon the particular genotypes of the inbred lines to be crossed. These must give rise to a propitious heterozygous condition in which specific genetic determinants promote positive effects through dominance or over dominance interactions. Heterosis too is not maintained in the F2 and subsequent generations owing to the decrement of heterozygosity in part of the offsprings. So, both luxuriance and heterosis represent short-term genetic gains, in the sense that they are attainable in the first progeny and cannot be improved further. Their immediate appearance, however, does not mean that they canb e achieved quickly because to find the right combinatoion of species for luxuriance or inbred lines for heterosis may take quite some time.

Monosexuality is sometimes exhibited by interspecific hybrids and may be exploited whenever the exclusive sex performs better in culture than the other. All female hybrids were produced from the cross between female *M. saxatilis* X male *M.mississippiensis*. All male hybrids can be obtained by crossing different tilapia species but their use on a large scale has not been entirely successful, as females reappear in later progenies because of broodstock infiltration by hybrids. Thus, tilapia hybridization has been largely replaced by direct hormonal masculinization. The use of androgenetic YY males to produce all or nearly all male (XY) progeny in crosses with normal XX females has been also proposed as a valid alternativein the Nile tilapia.

Novelty consists in the appearance in the hybrid of a positive trait that was not present in the parental species. An example is provided by the appealing marbled colour pattern of the so-called "tiger" trout, the hybrid of the cross between female brown trout *(Salmo trutta)* X male brook trout *(Salvelinus fontinalis),* that was superior in culture to its reciprocal and triploid variants. lnterspecific and intergeneric hybridization in marine fish culture Hybridization experiments in marine teleosts are still limited in number as compared to freshwater species but indicate that, especially in the family's Moronidae and Sparidae, hybrids may have a considerable potential for cornmercial culture.

7. Genetic engineering

Following the pioneering experiment concerning the transfer of a growth hormone (GH)-encoding genetic construct into the fertilized eggs of the goldfish to enhance body growth, a vast body of literature has accumulated on the topic of transgenesis in teleost fish. In broad terms, gene transfer in fish is exploited in both basic and applied research.

7.1 Fish transgenesis in basic research

In fundamental science, gene transfer in fish is a valuable tool in the study of the regulation of gene expression, particularly in the areas of developmental biology and evolution of functional genomics, and relies chiefly on two aquarium fishes as experimental models, namely the zebrafish and the medaka.

The main advantages of these fish (and other similar model fishes, such as platy fish and swordtails) for gene transfer as compared to mammalian systems, like transgenic mice, are: (i) the large and continuous availability of gametes and embryos for direct manipulation at a low cost; (ii) the relative rapidity of embryonic development; and (iii) the transparency of embryos, pre-larvae and larvae (up to the pigmentation stage) for direct localization of reporter gene transcription. These features make these fish very handy especially fo *in vivo* transient gene expression assays.

Conversely, there are also some disadvantages with piscine systems that still need be overcome because they lower considerably the transgene integration efficiency and hinder the production of stable transgenic lines and transgene targeting within the recipient genome. The first obstacle is the yolk content of fertilized eggs that prevents pronuclear microinjection, as it is routinely done in the mouse. Hence, transgenes must be introduced into the ooplasmic compartment of postovulatory oocytes and fertilized eggs or into the blastomeric cytoplasm of early segmenting embryos using a variety of techniques (microinjection through the chorion or micropyle, electroporation, particle gun bombardment, and transfection with liposomes). This approach requires the use of a much higher number of transgene copies (10^5-10^7 copies/cell instead of 200 copies/pronucleus, as in the mouse) to favour nuclear incorporation and chromosomal integration. Despite this compensation, transgene integration tends to be retarded and its efficiency rather low in fish.

At present, medaka is the only teleost in which foreign DNA can be successfully injected into the polarized germinal vesicle of primary oocytes, but this techniques involves complex procedures and still requires $5x10^3$-10^4 transgene copies nucleus. Effectiveness of gene transfer mediated by sperm cells subjected to incubation or electroporation with the exogenous DNA, though claimed in zebrafish, seems to be very low or negligible, as observed also in mammals.

The second obstacle is the extremely rapid succession of the first 10-11 cleavages in fish embryos without G1 and GP phases and virtually no transcription up to the mid-blastula stage. This fast segmentation presumably supported by a large store of proteins and precursors for DNA replication and chromatin assembly that is not available in mammals, whose early development is much slower.

This state of affairs tends to promote: (i) replication of incorporated DNA, mostly in an extrachromosomal state, with an overall increasein the number of copies nucleus; (i) concatemerization (i.e formation of tandem arrays) of incorporated and replicated exogenous DNA sequences carried out by stored ligases, whose activity is likely stimulated by their high

concentrations; (iii) mosaicism of expression and chromosomal integration, if any, of each single transgene within the embryo as a result of unequal initial nuclear incorporation and differential replication, degradation or subsequent fate of transgene copies; (iv) transgene silencing by DNA methylation and heterochromatin formation that seems to be more intense when integration occurs as multiple-copy concatamers and is probably responsible for the fading away of transgene expression in successive fish generations.

These drawbacks prevent the non-mosaic integration of the transgene as a single intact copy as opposed to concatamers. Moreover, they hinder the application of two-step transfection techniques, such as the use of cultured pluripotent embryonal stem (ES) cells as foreign DNA vectors in mice, to perform transgene targeting, like gene knockout and gene replacement through homologous gene recombination. Although ES cell lines can be derived from blastula-stage embryos of zebrafish and grown in culture, where they can be transfected and selected for the desired copy number, expression level and genomic localization of the transgene, their transplantation into premid-blastula stage fish embryos to give rise to chimeric transgenic somatic tissues and germline is not as straightforward as in mice. In fact, the proliferation rate of transplanted ES cells is much slower than that of the cells in the recipient embryo, probably because they are not endowed with the same afore mentioned replicative machinery, and their competition to contribute to the total cells of the embryo is consequently curtailed. Since later on only a small number *of* gametes will be transgenic, if any, the production and identification of transgenic progeny becomes cumbersome.

Alternative approaches are worth exploring to improve the control on gene transfer in fish to a level comparable to the much more successful research with transgenic mice. A promising avenue would be the transplantation of single decondensed sperm nuclei, transfected by restriction enzyme-mediated integration of the transgene, into unfertilized eggs. This technique has been fruitfully applied in *Xenopus laevis* to produce embryos expressing the transgene integrated in a few copy number (as single copy or short (2-6 copy) concatamers), nonmosaically and with good tissue-specificity or regional restriction. This method yields a transgenic progeny with the transgene integrated in a hemizygous condition at different genomic sites that can be upgraded to a homozygous condition, at least at a single integration site, in part of the subsequent offspring obtained by normal reproduction. This process can be speeded up by resorting to reproduction by mitogynogenesis or diploid androgenesis instead of biparental breeding so to generate at once completely homozygous transgenic animals serving as progenitors of clonal stabilized transgenic lines. A further conceivable extension of the nuclear transplantation technology would be the microinjection of single nuclei from *in vitro* transfected and selected ES cell into genetically inactivated eggs to produce non-chimeric transgenic fish bearing targeted transgenes. Homozygosityo f transgenes could thenb e achieved later on by unisexual or bisexual reproduction. These developments, if implemented, would eliminate the present shortcomings of ES cell technology in fish and merge eventually into a technology for the transfer of nuclei from a variety of somatic cell types stably transfected in culture. The latter approach was used to produce transgenic sheep, because in this species ES cells capable of contributing to the germline have not yet been isolated.

7.2 Gene transfer to improve production in fish culture

Transgenesis aimed to benefit fish culture has been mainly performed in freshwater-spawning teleosts, that is cyprinids, salmonids, cichlids, esocids and ictalurids (cf., above reviews). Most experiments have concentrated on growth enhancement by microinjection of

GH-encoding genetic constructs into fertilized eggs, but research has also been directed towards increasing the freeze resistance of Atlantic salmon and cold tolerance of goldsfish as well as to develop a transgenic approach to confer disease resistance.

In addition to the difficulties and problems discussed above regarding gene transfer in fish in general, three other issues need be considered when dealing with transgenic fish for massive culture, namely environmental safety, economic convenience and consumer acceptance.

Environmental safety

The problem of safety has been repeatedly raised and can be best overcome by rearing a limited number of transgenic broodfish in closed systems with recirculating water to ensure complete physical confinement, and by growing for fattening only totally sterile fish produced by interspecific hybridization, triploidy induction, genetic sterility induced by homozygous recessive alleles, expression of a GnRH anti-mRNA driven by a GnRH gene promoter or other equivalent means. Risk assessment studies, based on theoretical inference or even pilot release trials, to figure out the possible undesirable environmental impacts of fertile transgenic fish set loose into the wild, have uncertain predictive value and applicability and may actually cause dispersal of transgenic fish.

Nevertheless, a strong stand against the assumption that the release of genetically modified fish, including warm water marine fish, like the gilthead seabream, that have a great potential for dispersal, a low probability for recapture and a reasonable chance to survive and reproduce in the wild, may in any way represent an implant of superior variants capable of genetic amplification at the expense of feral conspecifics and other aquatic populations. On the opposite, based on the previous ecologically risk-free record of genetically engineered domestic plants and terrestrial animals, he contends the hypothesis that laboratory-induced genetic changes "have a negligible probability of being selectively favoured in wild populations under natural selection, and accordingly, without sustained large releases, have little potential for ecological impact". The crucial argument is that without substantial knowledge of the complexities in genomic architecture and gene interplay required for phenotypic adaptation to the environment, any engineered genetic change is fated to exalt a peculiar trait at the detriment of overall fitness in the wild, in a similar way as classical random mutations.

This reasoning is well substantiated by existing evidence and may be endorsed to remove excessive concern about controlled research on gene transfer in fish and the setting up of transgenic broodstocks of a limited size under physical confinement. This policy would be analogous to the official guidelines for gene transfer in the mouse, a cosmopolitan pest species with thousands of transgenics already produced, none of which has consolidatedi n the wild.

Conversely, reliance on a precautionary attitude towards the mass cultivation near or at the sea of non-sterile transgenic marine fish is likely to prevail at present, justified by lack of pertinent data and our scarce control over the marine environment. Restrictive regulations will then continue to be enforced, unless one of the following conditions are met: (i) conclusive proof that non-sterile transgenic fish cannot propagate in the wild; (ii) demonstration of the complete and permanent sterility of transgenic fish for on growing; or (iii) fattening of marine transgenic fish in farms operating with recirculating systems in the

hinterland, away from the sea, in the case that the previous two conditions have not been convincingly fulfilled.

Economic convenience

As to economic convenience, the question of whether it might be profitable for the producers in the Mediterranean region to invest in transgenic technology can be answered at this time only in general terms owing to the fact that interest in this opportunity is merely incipient. Yet, a number of possible advantages can be easily put on a list, such as: (i) improvement of disease resistance in transgenics would be a valuable trait, considering the economic losses caused by recurrent epizootics in marine fish culture; (ii) enhancement of growth at the larval and perimetamorphic stages may reduce the considerable costs of the current rearing technology, based on the prolonged administration of living preys, by anticipating the time of weaning with an inert diet; (iii) amplification of anabolism in fast-growing transgenics may ameliorate the conversion efficiency of high energy feed, like extruded pellets with a high lipid content, into a lean filet meat, thus reducing unwanted accumulation of intramuscular and perivisceral fat; an increase in food conversion efficiency (up to 30%) by administered recombinant fish GHs has been repeatedly reported; (iv) fast-growing transgenics are best suited for culture in recirculating systems where the benefits of optimized culture conditions, protection from pathogens and negligible polluting discharges are counteracted by high running costs that can be sustained only if the production cycle is considerably shortened; (v) genetic engineering of cultured carnivorous species to confer additional digestive and metabolicapabilities (euryphagy) may allow feeding on less expensive nutrients, such as carbohydrates or vegetable oils, thus partially replacing animal protein and fish oil in the diet; (vi) the ownership of a transgenic broodstock is likely to afford a control over a share of the fish market that is expected to grow larger and larger, the lower the price of the marketed transgenic fish; and (vii) expertise gained in the transgenic culture of Mediterranean fish species may be put to use in co-operative programs with developing countries centred on other species, such as cyprinids and cichlids, that are important for a multitude of consumers with a limited supply of animal protein.

Consumer acceptance

With regard to consumer acceptance, this is likely to evolve together with the public perception of genetically modified organisms as a result of the combined conflicting influence of traditional values, ethical issues, ideological positions, political mood, mass media orientation in information processing and science popularization and other unpredictable variables. Because there is no need for a transgenic technology in marine fish culture unless it can supply a product of superior quality at a lower price, a transition in consumers' attitude towards transgenic fish can then be envisioned, on the basis of sheer convenience, from initial refusal to tolerance and, finally, to appreciation. A similar trajectory can be recognized in the course of the last twenty-five years with respect to cultured vs. captured seabass and seabream. With a drop in retail price to about one third of what was offered in the past, and the general good uniformity and dependable quality of the cultured product, few people really care nowadays whether the fish on sale had lived in a floating cage instead of struggling for life on the high seas. Actually, with a deepening perception of the existential value of wild life, it may be reassuring, if not comforting, to know that one's meal demanded the sacrifice of a creature born for that purpose and grown with care.

However, to believe that consumers' choices are motivated exclusively by a rational appraisal of sheer convenience is an oversimplification, as diffidence towards novelty is often

a critical barrier to food product acceptance. This meant that the marketing strategy for genetically engineered fish must concentrate not only on an appealing quality/price ratiob ut also on gradualness, moderation and fairness. Gradualness consists in offering to consumers a series of intermediate products with increasing divergence with respect to consolidated items to soften the impact of novelty. A conceivable sequence may be: triploid and monosexual fish + sterile hybrids cultured in open or closed systems *3* diversified sterile hybrids of unimaternal/polypaternal origin + disease-resistant sterile transgenics + fast growing sterile transgenics + double-muscled sterile transgenics (see below) + euryphagic sterile transgenics and so on of course, some of these novel traits could be engineered not only in parallel but also in series along the same transgenic line.

Presumably, only a part of the above sequence would be really implementable in Mediterranean cultured fish species, whose market volume is probably not big enough to justify huge research investments, if it were not for the fact that the same biotechnology may be profitably extended to other species of interest for third world countries. Another point to take into account is that quantitative genetics is also being applied to fish to pursue some of the same goals as transgenetics, like disease resistance and growth enhancement. At present, it cannot be predicted what approach will meet with more success, whether the consolidated methodology of quantitative genetics with its onerous logistics and gradual gains, or the technological dynamism of transgenetics with its present experimental diversification and search for sudden breaktroughs. The requirement of moderation in cultured fish transgenesis refers to the recommendation of refraining from too radical genetic changes induced by transgenes with an overly artificial design. So far, all experiments have been aimed towards the direct phenotypic expression of gene constructs, whereas the possible benefits of knocking out the expression of constitutive genes by homologous recombination has not yet been investigated for lack of an adequate technology, as discussed above. In this respect, an attractive target might be the myostatin gene, if present in fish, whose inactivation by a random mutation - an 11-bp deletion – gave rise to the double-muscled Belgian Blue bovine strain. Mice with a disrupted myostatin gene were significantly larger than wild-type animals and showed a 2-3 times increasein skeletal muscle mass. A transgenic fish with a better performance in culture because it bears a suppressed gene is likely to be more acceptable by the food market as compared to a fish actively expressing a transgene.

Most research on gene transfer to improve growth in farmed fish is now conducted with transgenes containing exclusively fish-derived sequences ("all-fish" constructs) in place of the mammalian, avian and viral regulatory and coding sequences used in early work. This positive trend towards a greater homology between the transgene and the recipient genome has not yet progressed further into the selection of more finely tuned promoters to drive the expression of complementary or genomic hormone-encoding DNAs. In fact, the pursuit of high expression levels of GH has prompted the use of chimeric constructs containing regulatory sequences with either a widespread tissuexpression, such as the salmonid metallothionein gene promoters and the carpß -actin gene promoter, or with a tissue-specific expression of high intensity, such as the hepatotropic oceanpout antifreeze protein gene promoters. Unfortunately, the overexpression of GH is not always correlated with a better growth.

Oncorhynchus kisutch, bearing a construct consisting of the sockeye salmon GH genef used to the sockeye salmon metallothionein-B promoter, were on average 11-fold heavier (and some individuals up to 37-fold heavier!) than non-transgenic controls at 14 months of age and had GH plasma levels up to 40-fold higher due to an uncontrolled overexpression of

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the transgene. These fish were affected by various abnormalities, such as acromegaly, cranio-facial deformities and opercular overgrowth, and showed reduced viability. Recently, transgenic Nile tilapias, carrying a chinook salmon GH gene driven by an ocean pout antifreeze protein promoter, were characterized by a high level of exogenous GH expression and a 4 times greater growth rate than their non-transgenic full siblings. Transgenic males, however, were subfertile or completely sterile. These and similar results suggest that a convenient alternative might be the transfer of GH gene constructs driven by their own regulatory sequences to target expression to the pituitary, because the increase of GH levels in the resulting transgenics would be more physiologically controllable. Oddly enough, gene transfer experiments of this kind have never been performed so far in farmed fish, probably for the fear that internal negative feedback mechanisms might depress the expression of the transgene. Consequently, basic research on the transcriptional control of the GH gene in fish has not contributed at all to transgenesis applied for growth enhancement in fish culture.

Investigation on the functional organization of the promoter region of the GH2 gene of rainbow trout (rtGH2) as compared to those of mammals has revealed that the cis- and tans-regulatory elements governing GH expression are highly conserved in vertebrates and that they evolved essentially combinatorially (i.e., by different assortments and positioning of the same regulatory units within the various promoters) rather than molecularly (i.e., through the development of novel regulatory units) according to the contingent adaptive needs of each species. Another interesting aspect is that the proximal promoter region of rtGH2, stretching across about 200 bp 5' upstream of the transcription start, is sufficient to confer a strong tissue-specific expression to a reporter gene in transfection assays. In fact, this region contains 3 binding sites (FI-F3) for the conserved, pituitary restricted transcription factor Pit-l, which is required for the expression in somatotropic cells.

Moreover, the region encompassing the F2 and F3 Pit-l-binding sites includes two additional regulatory elements: a TGACG motif, corresponding to CRE (CAMP response element), that stimulates transcription upon interaction with the trans-factor CREBP (CRE-binding protein) once phosphorylated by protein kinaseA ; and a GRE like (glucocorticoid response element) motif that activates transcription after binding with the saturated corticosteroid receptor. These two elements confer a synergistic responsiveness to CAMP and cortisol on the rtGH2 promoter. Interestingly, the promoter region located upstream of the F3 site is apparently inhibitory, even though it carries an additional Pit-l-binding site (F4).

These data suggest that the proximal promoter is essentially involved in transcription activation whereas other regulatory elements with either enhancer or silencer function may be located further upstream or elsewhere. Hence, the use of constitutive promoters of short length should ensure the eutopic expression of GH encoding transgenes without responsiveness to internal negative feed-back mechanisms. Longer promoters may favour instead greater modulatory control. Moreover, native promoters could be modified not only in length but also in the arrangement of their response elements, whose number and position may be changed.

Fish expressing GH-encoding transgenes eutopically or even ectopically but at a low level, may better reconcile growth enhancement with the preservation of a normal phenotype. In Mediterranean cultured fish, more physiological promoters could be coupled to species-specific GH encoding DNAs, although this may complicate somehow the assessment of transgene integration. In fact, the cDNA sequence of GH has been determined in both seabream and seabass. Transgenic fish bearing species-specific constructs with eutopic

expression, if produced in the future, would be autotransgenic and might be even considered for use as broodstockin selective breeding programs. In this case, transgenetics would blend with quantitative genetics.

Finally a brief remark about fairness in fish transgenesis, an issue that amounts to whether and how to inform consumers about fish have been genetically engineered. As for other transgenic products, an obvious marketing strategy is to avoid any publicity on the grounds that only transgenic fish that are nutritionally safe can be approved for sale. Because this statement is reasonably true, any label attesting the type of production technology seems indeed redundant and possibly a source of confusion and anxiety on the part of the consumers. Yet, this approach is unfair. It is manipulative, opportunistic and intimately distrusting both the product and the consumer. Perhaps one day, we shall realize that we all belong to a society of consumers and that we deserve respectful information merely on transgenics but on any product.

7.3 Conclusion

In conclusion, aquatic biotechnology is expected to provide a fundamental contribution to the foreseeable development of fish culture. In this regard, the Mediterranean cultured fish represent a stimulating challenge for two main reasons: their biology is more difficult to approach than that of freshwater fishes and thus requires a more sophisticated technological level; and any achievement in these species is likely to open new avenues for the expansion of mariculture and the appreciation of its enormous potential for mankind. In this endeavour, the scientific community needs not only the ingenuity, dedication, prudence and internal criticism of its members but also an informed perception and support by the general public.

There is a tremendous beauty concealed in marine life with its generous trove of gifts to our existence. Aquatic biotechnology is an attempt to enrich this beauty with a small sign of human intelligence, perhapsto enlarge the significance of our fleeting time, more probably to watch ourselves as a part of a miracle.

8. Artificial propagation for Chromosome manipulation

Manipulation of fish reproduction, an important scientific development for aquaculture, may be performed at various levels of artificial propagation. Spawning may be controlled through environmental manipulation or through direct hormonal intervention. The basic techniques and capacity for control vary between species. Tilapias do not respond well to hormonal induction but can be induced to spawn through manipulation of environmental variables. A suitable temperature and controlled photoperiod permit a reasonably close prediction of ovulation time.

Chromosome manipulation, which includes ploidy alteration or euploidy induction with single parent genome contribution, requires the collection of freshly released gametes. Ploidy manipulation includes the induction of triploids through polar body retention or tetraploidy through interference with first karyokinesis. Euploid alterations include gynogenetic (gynogenote) diploidization of the maternal genome through polar body retention (meiogynote) or mitotic interference (mitogynote). Diploidization of the paternal genome (androgenote), or androgenesis, is accomplished by interference with first mitosis. In the case of androgenesis and gynogenesis, either the female or male DNA, respectively, must be

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rendered inactive before the egg is activated. Both parental genomes are intact, however, during ploidy manipulation.

Chromosome manipulation involves one or two basic treatments of freshly obtained gametes. For gynogenesis or androgenesis the first treatment is the genome deactivation of the spermatozoa or eggs, respectively. Ultraviolet irradiation is preferred for simplicity and safety, but also because it dimerizes the DNA rather than fragmenting it. Activation of treated spermatozoa or eggs requires diploidization by some form of shock that either retains the second polar body (pb) or interrupts the first mitotic karyokinesis. Shock is most often physical (e.g., thermal [cold or hot] or pressure). Thermal treatment is usually preferred because of the ease of application and the simplicity of the equipment used. To disrupt chromosome separation the shock must coincide with metaphase and be sufficiently severe to disrupt the spindle fibers. Thus, shock intensity, duration, and time of application must be optimally combined to ensure a maximum yield of progeny. Further, because the rate of development is inversely temperature dependent, either the preshock incubation temperature must be standardized or the shock time must be calibrated to compensate for the temperature effect. Absolute shock time (τ_S, in minutes post-activation) can be related to tau (τ_0, in minutes) to report shock protocol in a dimensionless term (τ_S/τ_0) which is temperature compensated.

Spawning

Spawning of *O. niloticus*, was managed by photoperiod manipulation. Four to six females were stocked with one male in four 550l aquaria. Water was aerated and circulated at the rate of one turnover per day and temperature was maintained at 26 ˚C. The light cycle was initiated at 1h; the time of sunset signalled the end of the photoperiod for a total of about 20 light hours and 4 dark hours (20L:4D). *O. niloticus* spawns about 8 to 10 h after the beginning of the light cycle. Ovulation and spawning readiness were determined through observation of courtship behavior, coloration, and papilla erection. Females were stripped after the initiation of spawning or upon observation of other determinants.

Fertilization

Eggs were collected in a clean container, milt was expressed over the eggs, and water was directly added to initiate activation. Within two to three minutes of activation fertilized eggs were placed in controlled temperature (0.2˚C) incubators. The incubation temperature was closely regulated to document the embryonic rate of development, so that timing of diploidization shock could be determined. Eggs were incubated in 1-l upwelling units with sufficient flow to gently tumble the developing embryos.

Tau Estimates

Developmental rate is defined as the duration of one mitotic cycle during early synchronous cleavage. The mean interval between the initiation of the first and third mitoses in 5 to 10% of the eggs was recorded at temperatures within the usual developmental range (20 to 30°C). Twenty to thirty eggs were examined under magnification at five-minute intervals. Tau curves have been used to facilitate chromosome manipulation studies in various fishes. The mechanics of this technique have been more difficult with tilapias due to the difficulties associated with gamete collection.

Results

Photoperiod manipulation induced spawning activity to occur at midday. Females ovulated/ spawned between 9.5 and 13.5 h (mean = 10.1 h) after initiation of the light cycle. There was no apparent correlation between the latent period and water temperature; however, temperature variation was low (23.9 to 27.7˚C). During the spawning season, eggs were collected from ten Nile tilapia. Fertilization rates were variable with no development in five of ten batches. Fertilization and hatching rates are poor among tilapias even though egg resorption is common. Egg resorption does not always prevent ovulation of the bad eggs. The low rate of fertilization may also be due to the type of incubation system used.

The hatch rate of an upwelling type of incubator is not as good as a down-flow system incubator. Estimates of tau were made at five temperatures ranging from 20.6 to 27.5˚C (Table 1). The sample size of this study is too small to evaluate variability; however, the estimates of variability have been tightly associated with a tau curve calculated from data on time to first mitosis in *O. aureus*. The theoretical tau curve for Nile tilapia used in this study was generated using the techniques of Rubinshtein *et al.* This curve is characterized by the relationship:

$$\tau_0 = 10^5 x^{-2.39}$$

where x is temperature in ˚C. The tau-temperature relationship showed an inverse correlation and ranged from 73.5 to 30 minutes in the observed temperature range.

Table 1. Temperature-tau relationship for Nile tilapia

Incubation Temperature (˚C)	Observed $\hat{\tau}_0$ (minutes)	Calculated $\hat{\tau}_0$ (minutes)
20.6	73.5	72
21.6	67.5	65
22.4	56.5	59
24.7	50	47
27.5	30	36

Calculated curve developed from data for time to first mitoses in *O. aureu* based on methods of Rubenshtein et al. (1997); the calculated tau curve is characterized by the relationship: $\tau_0 = 10^5 x^{-2.39}$ where x = temperature (˚C).

Discussion

Ovulation based on the light cycle was a reasonable means for predicting time of stripping. Hormonal induction of ovulation would seem to be a logical method for determination of egg collection; however, cichlids have responded poorly to gonadotropic therapy. Conversely, the physiological characteristics of tilapia gametes provide an advantage in chromosome manipulation. Eggs retain high fertility for three to six hours post-immersion in water and sperm remain motile in water for several hours in contrast with most fishes. Thus, the quality of gametes should not deteriorate during UV treatment in comparison with other fishes. More suitable incubation techniques must be incorporated in the chromosome manipulation protocol, because survival is lower for genomemanipulated progeny.

A reasonable first estimate of a tau curve can be used to standardize shock treatments. The next aspect of this research will be the development of UV treatment of eggs. Treatment of sperm with UV has been routine for the induction of gynogenesis but few studies have attempted androgenesis, which requires egg treatment. Even fewer studies have used UV in female genome deactivation. Preliminary trials have verified the general dosage level (400 to 600 J m^{-2}).

Thermal shock treatments will be used for practical reasons and because their effectiveness is comparable to or better than pressure shock. However, the timing for shock at 27 minutes post-activation (28˚C) contradicts the optimal shock time of 92 minutes post-activation (26˚C) even when adjusted for temperature. Therefore, these reported optimal times and shock types will be examined within the context of tau information.

A visual genetic marker is vital in chromosome manipulation studies. One option with Nile tilapia is the use of normal-color females and males of the blond mutant. This homozygous recessive trait would usually be appropriate. Alternatively, the red mutant, which is a dominant gene, could be used for an egg source and normal-color males as sperm donors. While developing the red mutant line, androgenetic experimentation shall proceed using the current Nile stock and a gold mutant of *O. mossambicus* as the sperm donor.

The development of androgenesis for *O. niloticus* should provide an alternative method for producing YY-males for the monosex production of tilapia.

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Chapter 19

Apiculture

1. Beekeeping

Beekeeping or apiculture is the maintenance of honey bee colonies, commonly in hives, by humans. A beekeeper (or apiarist) keeps bees to collect honey and beeswax, to pollinate crops, or to produce bees for sale to other beekeepers. A location where bees are kept is called an apiary.

There are more than 20,000 species of wild bees. Many species are solitary, and many others rear their young in burrows and small colonies, like mason bees and bumble bees. Beekeeping, or apiculture, is concerned with the practical management of the social species of honey bees, which live in large colonies of up to 100000 individuals. In Europe and America, the species universally managed by beekeepers is the Western honey bee (*Apis mellifera*). This species has several sub-species or regional varieties, such as the Italian bee (*Apis mellifera ligustica*), European dark bee (*Apis mellifera mellifera*), and the Carniolan honey bee (*Apis mellifera carnica*). In the tropics, other species of social bee are managed for honey production, including *Apis cerana*.

Figure 1: *Apis mellifera*

All of the *Apis mellifera* (Figure 1) sub-species are capable of inter-breeding and hybridizing. Many bee breeding companies strive to selectively breed and hybridize varieties to produce desirable qualities: disease and parasite resistance, good honey production, swarming behaviour reduction, prolific breeding, and mild disposition. The advantages of the initial F1 hybrids produced by these crosses include: hybrid vigor, increased honey productivity, and greater disease resistance. The disadvantage is that in subsequent generations these advantages may fade away and hybrids tend to be very defensive and aggressive.

At some point humans began to domesticate wild bees in artificial hives made from hollow logs, wooden boxes, pottery vessels, and woven straw baskets or "skeps."

For several thousand years of human beekeeping, human understanding of the biology and ecology of bees was very limited and riddled with superstition and folklore. Ancient

observers thought that the queen bee was in fact a male, called "the king bee," and they had no understanding of how bees actually reproduced. It was not until the 18th century that European natural philosophers undertook the scientific study of bee colonies and began to understand the complex and hidden world of bee biology. Swammerdam and Réaumur were among the first to use a microscope and dissection to understand the internal biology of honey bees. Réaumur was among the first to construct a glass walled observation hive to better observe activities within hives. He observed queens laying eggs in open cells, but still had no idea of how a queen was fertilized; nobody had ever witnessed the mating of a queen and drone and many theories held that queens were "self-fertile," while others believed that a vapor or "miasma" emanating from the drones fertilized queens without direct physical contact. Huber was the first to prove by observation and experiment that queens are physically inseminated by drones outside the confines of hives, usually a great distance away.

Following Réaumur's design, Huber built improved glass-walled observation hives and sectional hives which could be opened, like the leaves of a book, to inspect individual wax combs; this greatly improved the direct observation of activity within a hive. Although he became blind before he was twenty, Huber employed a secretary, Francois Burnens, to make daily observations, conduct careful experiments, and to keep accurate notes over a period of more than twenty years. Huber confirmed that a hive consists of one queen who is the mother of all the female workers and male drones in the colony. He was also the first to confirm that mating with drones takes place outside of hives and that queens are inseminated by a number of successive matings with male drones, high in the air at a great distance from their hive. Together, he and Burnens dissected bees under the microscope and were among the first to describe the ovaries and spermatheca, or sperm store, of queens as well as the penis of male drones. Huber is universally regarded as "the father of modern bee-science" and his "Nouvelles Observations sur Les Abeilles (or "New Observations on Bees") revealed all the basic scientific truths for the biology and ecology of honeybees.

2. Natural Beekeeping

There is a current movement that eschews chemicals in beekeeping, and feels that colony collapse disorder can be most effectively addressed by reversing trends that disrespect the needs of the bees themselves. Crop spraying, unnatural conditions in which bees are moved thousands of miles to pollinate commercial crops, artificial insemination of queens, and sugar water feeding are thought to all contribute to a general weakening of the constitution of the honeybee.

"Since the honey bee exerts such a powerfully beneficial influence on the natural world around us, it seems logical to assume that our own efforts to help the honey bee thrive can indirectly benefit all of nature."

3. Bee colonies

3.1 Castes

A colony of bees consists of three castes of bee:

- a Queen bee, which is normally the only breeding female in the colony;
- a large number of female Worker bees, typically 30,000–50,000 in number;
- a number of male drones, ranging from thousands in a strong hive in spring to very few during dearth or cold season.

The queen is the only sexually mature female in the hive and all of the female worker bees and male drones are her offspring. The queen may live for up to three years or more and may be capable of laying half a million eggs or more in her lifetime. At the peak of the breeding season, late spring to summer, a good queen may be capable of laying 3,000 eggs in one day, more than her own body weight. This would be exceptional however; a prolific queen might peak at 2,000 eggs a day, but a more average queen might lay just 1500 eggs per day. The queen is raised from a normal worker egg, but is fed a larger amount of royal jelly than a normal worker bee, resulting in a radically different growth and metamorphosis. The queen influences the colony by the production and dissemination of a variety of pheromones or 'queen substances'. One of these chemicals suppresses the development of ovaries in all the female worker bees in the hive and prevents those laying eggs.

3.2 Mating of queens

The queen emerges from her cell after 15 days of development and she remains in the hive for 3-7 days before venturing out on a mating flight.Mating flight is otherwise known as 'nuptial flight'. Her first orientation flight may only last a few seconds, just enough to mark the position of the hive. Subsequent mating flights may last from 5 minutes to 30 minutes, and she may mate with a number of male drones on each flight. Over several matings, possibly a dozen or more, the queen will receive and store enough sperm from a succession of drones to fertilize hundreds of thousands of eggs. If she does not manage to leave the hive to mate-possibly due to bad weather or being trapped within part of the hive - she will remain infertile and become a 'drone layer', incapable of producing female worker bees, and the hive is doomed.

Mating takes place at some distance from the hive and often several hundred feet up in the air; it is thought that this separates the strongest drones from the weaker ones - ensuring that only the fastest and strongest drones get to pass on their genes.

3.3 Female worker bees

Most bees in a hive are female worker bees. At the height of summer when activity in the hive is frantic and work goes on non-stop, the life of a worker bee may be as short as 6 weeks; in late autumn, when no brood is being raised and no nectar is being harvested, a young bee may live for 16 weeks, right through the winter. During its life a worker bee performs different work functions in the hive which are largely dictated by the age of the bee.

Period	Work activity
Days 1-3	Cleaning cells and incubation
Day 3-6	Feeding older larvae
Day 6-10	Feeding younger larvae
Day 8-16	Receiving honey and pollen from field bees
Day 12-18	Wax making and cell building
Day 14 onwards	Entrance guards; nectar and pollen foraging

3.4 Male bees (drones)

Drones are the largest bees in the hive at almost three times the size of a worker bee. They do not work, do not forage for pollen or nectar and are only produced to mate with new queens and fertilize them on their mating flights. A bee colony will generally start to raise drones a few weeks before building queen cells to supersede a failing queen or in preparation for swarming. When queen raising for the season is over, the bees in colder

climates will drive the drones out of the hive to die, biting and tearing at their legs and wings; the drones have become a useless burden on the colony which can no longer be tolerated.

3.5 Differing stages of development

Stage of development	Queen	Worker	Drone
Egg	3 days	3 days	3 days
Larva	8 days	10 days	13 days
Pupa	4 days	8 days	8 days
Total	15 days	21 days	24 days

3.6 Structure of a bee colony

A domesticated bee colony is normally housed in a rectangular hive body, within which eight to ten parallel frames house the vertical plates of honeycomb which contain the eggs, larvae, pupae and food for the colony. If one were to cut a vertical cross-section through the hive from side to side, the brood nest would appear as a roughly ovoid ball spanning 5-8 frames of comb. The two outside combs at each side of the hive tend to be exclusively used for long-term storage of honey and pollen.

Within the central brood nest, a single frame of comb will typically have a central disk of eggs, larvae and sealed brood cells which may extend almost to the edges of the frame. Immediately above the brood patch an arch of pollen-filled cells extends from side to side, and above that again a broader arch of honey-filled cells extends to the frame tops. The pollen is protein-rich food for developing larvae, while honey is also food but largely energy rich rather than protein rich. The nurse bees which care for the developing brood secrete a special food called 'royal jelly' after feeding themselves on honey and pollen. The amount of royal jelly which is fed to a larva determines whether it will develop into a worker bee or a queen.

Apart from the honey stored within the central brood frames, the bees store surplus honey in combs above the brood nest. In modern hives the beekeeper places separate boxes, called 'supers', above the brood box, in which a series of shallower combs is provided for storage of honey. This enables the beekeeper to remove some of the supers in the late summer, and to extract the surplus honey harvest, without damaging the colony of bees and its brood nest below. If all the honey is 'stolen', including the amount of honey needed to survive winter, the beekeeper must replace these stores by feeding the bees' sugar or corn syrup in autumn.

3.7 Annual cycle of a bee colony

The development of a bee colony follows an annual cycle of growth which begins in spring with a rapid expansion of the brood nest, as soon as pollen is available for feeding larvae. Some production of brood may begin as early as January, even in a cold winter, but breeding accelerates towards a peak in May (in the northern hemisphere), producing an abundance of harvesting bees synchronized to the main 'nectar flow' in that region. Each race of bees times this build-up slightly differently, depending on how the flora of its original region blooms. Some regions of Europe have two nectar flows: one in late spring

and another in late August. Other regions have only a single nectar flow. The skill of the beekeeper lies in predicting when the nectar flow will occur in his area and in trying to ensure that his colonies achieve a maximum population of harvesters at exactly the right time.

The key factor in this is the prevention, or skillful management of the swarming impulse. If a colony swarms unexpectedly and the beekeeper does not manage to capture the resulting swarm, he is likely to harvest significantly less honey from that hive, because he will have lost half his worker bees at a single stroke. If, however, he can use the swarming impulse to breed a new queen but keep all the bees in the colony together, he will maximize his chances of a good harvest. It takes many years of learning and experience to be able to manage all these aspects successfully, though owing to variable circumstances many beginners will often achieve a good honey harvest.

4 Formation of new colonies

4.1 Colony reproduction; swarming and supersedure

All colonies are totally dependent on their queen, who is the only egg-layer. However, even the best queens live only a few years and one or two years longevity is the norm. She can choose whether or not to fertilize an egg as she lays it; if she does so, it develops into a female worker bee; if she lays an unfertilized egg it becomes a male drone. She decides which type of egg to lay depending on the size of the open brood cell which she encounters on the comb; in a small worker cell she lays a fertilized egg; if she finds a much larger drone cell she lays an unfertilized drone egg.

All the time that the queen is fertile and laying eggs she produces a variety of pheromones which control the behavior of the bees in the hive; these are commonly called 'queen substance' but in reality there are various different pheromones with different functions. As the queen ages she begins to run out of stored sperm and her pheromones begin to fail. At some point, inevitably, the queen begins to falter and the bees will decide to replace her by creating a new queen from one of her worker eggs. They may do this because she has been damaged (lost a leg or an antenna), because she has run out of sperm and cannot lay fertilized eggs (has become a 'drone laying queen') or because her pheromones have dwindled to a point where they cannot control all the bees in the hive anymore.

At this juncture the bees will produce one or more queen cells by modifying existing worker cells which contain a normal female egg. However, there are two distinct behaviors which the bees pursue:

- Supersedure: queen replacement within one hive without swarming
- Swarm cell production: the division of the hive into two colonies by swarming

Different sub-species of *Apis mellifera* exhibit differing swarming characteristics which reflect their evolution in different ecotopes of the European continent. In general the more northerly black races are said to swarm less and supersede more, whereas the more southerly yellow and grey varieties are said to swarm more frequently. The truth is complicated because of the prevalence of cross-breeding and hybridization of the sub species and opinions differ.

Supersedure is highly valued as a behavioral trait by beekeepers because a hive that supersedes its old queen does not swarm and so no stock is lost; it merely creates a new

queen and allows the old one to fade away, or alternatively she is killed when the new queen emerges. When superseding a queen the bees will produce just one or two queen cells, characteristically in the center of the face of a broodcomb (Figure 2).

In swarming, by contrast, a great many queen cells are created -typically a dozen or more -and these are located around the edges of a broodcomb, most often at the sides and the bottom.

Figure 2: New wax combs between basement joists

Once either process has begun, the old queen will normally leave the hive with the hatching of the first queen cells. When she leaves the hive the old queen is accompanied by a large number of bees, predominantly young bees (wax-secreters), who will form the basis of the new hive. Scouts are sent out from the swarm to find suitable hollow trees or rock crevices and as soon as one is found the entire swarm moves in, building new wax brood combs within a matter of hours using the honey stores which the young bees have filled themselves with before leaving the old hive. Only young bees can secrete wax from special abdominal segments and this is why there tends to be more young bees than old in swarms. Often a number of virgin queens accompany the first swarm (the 'prime swarm'), and the old queen is replaced as soon as a daughter queen is mated and laying. Otherwise, she will be quickly superseded in their new home.

4.2 Factors that trigger swarming

It is generally accepted that a colony of bees will not swarm until it has completed all its brood combs, i.e., filled all available space with eggs, larvae and brood. This generally occurs in late spring at a time when the other areas of the hive are rapidly filling with honey stores. So one key trigger of the swarming instinct is when the queen has no more room to lay eggs and the hive population is becoming very congested. Under these conditions a prime swarm may issue with the queen, resulting in a halving of the population within the hive and leaving the old colony with a large amount of hatching bees. The queen who leaves finds herself in a new hive with no eggs, no larvae but lots of energetic young bees who create a new set of brood combs from scratch in a very short time.

Another important factor in swarming is the age of the queen. Those under a year in age are unlikely to swarm unless they are extremely crowded, while older queens have swarming predisposition.

Beekeepers monitor their colonies carefully in spring and watch for the appearance of queen cells, which are a dramatic signal that the colony is determined to swarm.

When a colony has decided to swarm, queen cells are produced in numbers varying to a dozen or more. When the first of these queen cells is sealed, after 8 days of larval feeding, a virgin queen will pupate and be due to emerge seven days after sealing. Before leaving, the worker bees fill their stomachs with honey in preparation for the creation of new honeycombs in a new home. This cargo of honey also makes swarming bees less inclined to sting and a newly issued swarm is noticeably gentle for up to 24 hours — often capable of being handled without gloves or veil by a beekeeper.

Figure 3: A swarm attached to a branch

This swarm (Figure 3) is looking for shelter. A beekeeper may capture it and introduces it into a new hive helping to meet this need. Otherwise, it will return to a feral state, in which case it will find shelter in a hollow tree, an excavation, an abandoned chimney or even behind shutters.

Back at the original hive, the first virgin queen to emerge from her cell will immediately seek out to kill all her rival queens who are still waiting to emerge from their cells. However, usually the bees deliberately prevent her from doing this, in which case, she too will lead a second swarm from the hive. Successive swarms are called 'after-swarms' or 'casts' and can be very small, often with just a thousand or so bees, as opposed to a prime swarm which may contain as many as ten to twenty thousand bees.

Small after-swarms have less chance of survival and may deplete the original hive threatening its survival as well. When a hive has swarmed despite the beekeeper's preventative efforts, a good management practice is to give the depleted hive a couple frames of open brood with eggs. This helps replenish the hive more quickly, and gives a second opportunity to raise a queen, if there is a mating failure.

Each race or sub-species of honeybee has its own swarming characteristics. Italian bees are very prolific and inclined to swarm; Northern European black bees have a strong tendency to supersede their old queen, without swarming. These differences are the result of differing evolutionary pressures in the regions where each sub-species evolved.

4.3 Artificial swarming

When a colony accidentally loses its queen, it is said to be 'queenless'. The workers realize that the queen is absent after as little as an hour, as her pheromones fade in the hive.

The colony cannot survive without a fertile queen laying eggs to renew the population. So the workers select cells containing eggs aged less than three days and enlarge these cells dramatically to form 'emergency queen cells'. These appear similar to large peanut-like structure about an inch long, which hangs from the center or side of the brood combs. The developing larva in a queen cell is fed differently from an ordinary worker-bee, receiving in addition to the normal honey and pollen a great deal of royal jelly, a special food secreted by young 'nurse bees' from the hypopharyngeal gland. This special food dramatically alters the growth and development of the larva so that, after metamorphosis and pupation, it emerges from the cell as a queen bee. The queen is the only bee in a colony which has fully developed ovaries and she secretes a pheromone which suppresses the normal development of ovaries in all her worker-daughters.

Beekeepers use the ability of the bees to produce new queens to increase their colonies, a procedure called *splitting a colony*. To do this, they remove several brood combs from a healthy hive, taking care that the old queen is left behind. These combs must contain eggs or larvae less than three days old which will be covered by young 'nurse bees' which care for the brood and keep it warm. These brood combs and attendant nurse bees are then placed into a small 'nucleus hive' along with other combs containing honey and pollen. As soon as the nurse bees find themselves in this new hive and realise that they have no queen they set about constructing emergency queen cells using the eggs or larvae which they have in the combs with them.

5. Disruption of vitellogenin gene function in adult honeybees

Due to its social attributes, its learning capabilities and several facultative physiological and behavioural traits under social control, the honeybee (*Apis millifera*) provide unique opportunities as a model system. The honeybee has a rich history as an experimental organism, and it is now receiving increased attention concerning general issues such as the molecular basis of learning and memory generation, the regulation of ageing, the regulatory anatomy of social systems and the evolution of complex social systems through self-organization, emergence and multilevel natural selection. The ability to manipulate the genetics underlying the physiological and behavioural repertoires of the adult honeybee worker will become instrumental for understanding the above issues. However, there is currently no established method for doing clear-cut reverse genetics on adult bees.

One recent and very promising method for targeted down-regulation of gene expression in a wide range of organisms is RNAi. RNAi is the process by which dsRNA inhibits the accumulation of homologous transcripts from cognate genes. RNAi has evolved into a powerful tool for probing gene function in *Drosophila*, *Tribolium*, *Caenorhabditis elegans* and mice. In honeybees, microinjection in preblastoderm eggs using dsRNA derived from a 300 bp stretch of the engrailed homeobox motif disrupts expression of the target gene during embryo development. The adult honeybee gene expression exemplified by the gene coding for the lipoprotein vitellogenin can be specifically inhibited by microinjection of dsRNA into honeybee embryos as well as by intra-abdominal injection of adult bees.

The vitellogenin gene was chosen because its expression is unlikely to have a phenotypic effect until the adult stage in bees. The vitellogenin protein is produced by the adult insect fat body, which in honeybees consists of thin layers of cells spread against the body wall of the abdomen. Vitellogenin is normally secreted into the hemolymph of reproductive females before transport into developing oocytes. However, the sterile honeybee

workers may have high hemolymph titres of this protein. Vitellogenin mRNA is present in the fat body of bees < 24 hours old, while the level of corresponding protein in the hemolymph is undetectable at this age. The expression then increases, and the level of mRNA is constant in the age interval of 7–15 days. During this period the individual vitellogenin titre may vary greatly. This variation may be due to individual differences in the rate at which vitellogenin is consumed for metabolic purposes. Equal production rates would in this case give rise to variable titres. However, the phenomenon might also reflect individual differences in nutritional status, as the vitellogenin production is strongly dependent on the availability of proteinaceous food.

In any case, the vitellogenin titre is not an unambiguous assay for detecting RNAi. However, as the frequency of controls with negligible vitellogenin titres is low (7 % of all controls aged 5–15 days, N = 109), it is labour-saving to use it for detecting potential knockdown phenotypes. 15 % (N = 70) of the adult bees reared from eggs injected with dsRNA had strongly reduced levels of vitellogenin mRNA. The disruption appeared to be incomplete in some individuals, but the RNAi effect was detectable at emergence and persistent over 15 days. In the intra-abdominally injected group, 96 % (N = 30) showed loss of vitellogenin mRNA expression when sampled at 7 days old. Furthermore, an unambiguous fragment with an apparent size similar to the template dsRNA (504 bp) was visualized in all adult injected bees with a mutant phenotype. Samples enriched with short RNA show that vitellogenin mRNA fragments as small as 25 bp could be detected. For all mutants, the level of vitellogenin in the hemolymph was almost undetectable. Reduced levels of vitellogenin mRNA was not encountered in any of the control individuals assayed in this manner (N = 96).

Vitellogenin is a female specific glucolipoprotein yolk precursor produced by all oviparous animals. Invertebrate and vertebrate vitellogenins constitute a multigene superfamily together with insect apolipophorin II/I, human apolipoprotein B (apoB-100), and the large subunit of mammalian microsomal triglyceride transfer protein (MTP). It is generally synthesized in large amounts directly prior to yolk deposition and is of fundamental importance in reproduction. As vitellogenin is also synthesized by the functionally sterile workers, it has been suspected for some time that vitellogenin has functions other than reproduction in honeybees. It was recently shown that nurse bees use vitellogenin to produce royal jelly. Furthermore, vitellogenin serves important functions related to immune function and longevity. As the amount of vitellogenin mRNA in the honeybee worker is considerable, it can be suggested that RNAi can be used to also knock down abundantly expressed genes.

It cannot be excluded that the observed high efficacy of the intra-abdominal injection technique for inhibiting vitellogenin expression is due to the structure and function of the honeybee fat body. When dsRNA is injected into the honeybee abdomen, the fat body tissue is readily exposed. Furthermore, the fat body plays an immunological role that includes uptake of potentially harmful macromolecules from the hemolymph, in many ways analogous to the mammalian liver. The observed efficacy may thus not be representative for genes being expressed in other organs or tissues in the adult bee.

However, it is promising that this does not seem to be the case in *Drosophila melanogaster*. Intra-abdominal injections of homologous dsRNA in this species silence *lacZ* transgene expression in the gut as well as in the optic and antennal lobes, and that the method is potent in silencing endogenous GM06434 mRNA in the central nervous system. Even though the scope of this technique should turn out to be more restricted in honeybees,

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suggests that a number of important genes being expressed in the fat body can be targeted this way.

Alternatively, it may also be that the capacity of the fat body for taking up potentially harmful macromolecules from the hemolymph is indeed the key for targeting other tissues by intra-abdominal injection of dsRNA. If this dsRNA is processed into small interfering RNA (siRNA) or another mobile signal that is exported to the hemolymph, this would make the fat body a source of mobile silencing agents that probably could be more easily taken up by other tissues. Microinjections in the adult honeybee brain using dsRNA homologous to biogenic amine receptors results in incomplete knockdown of the target gene, are consistent with this explanation. If this suggestion is correct, the intra-abdominal injection technique may be superior to injecting dsRNA directly into a target tissue, both because of the actual mechanism, and the fact that the technique allows injection of much higher amounts of dsRNA without harming the individual.

Vitellogenin turn out to be representative for large number of genes being expressed in various tissues of the adult honeybee, the intra-abdominal injection technique provides a way to do functional genomics studies on adult honeybees by very simple means. The technique can be considered to be conditional to the extent that adult bees can be exposed to the dsRNA. It allows groups of genes to be simultaneously rendered ineffective without the need for time-consuming crosses, and it may even allow sequential targeted disruption that might turn out to be crucial for understanding certain types of regulatory structures. The technique can easily be extended to include delivery of siRNA. High-pressure injection of siRNA into the tail vein of postnatal mice has recently proven to be highly efficient for specific inhibition of transgene expression in a variety of organs. As use of siRNA seems to be a means to target specific isoforms in *Drosophila*, and to ensure a moderate longevity of RNAi in mice delivery of siRNA by intra-abdominal injection may provide additional possibilities for temporal and spatial control of RNAi in adult workers.

Ssubstantial RNAi in adults obtained from eggs injected with dsRNA at the preblastoderm stage, this injection method seems to provide a means to disrupt gene function in the embryonic, larval and pupal stages. However, intra-abdominal injection of dsRNA in adults is highly superior to egg injection for specific disruption of gene function in the adult stage. The intra-abdominal injection technique is much more efficient. It is substantially simpler, and it makes it possible to specifically address gene function in adults even in those cases where the focal gene is also expressed in the embryonic, larval or pupal stages.

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Chapter 20

Pearl Culture

1. Pearl

A pearl is a hard, generally spherical object produced within the soft tissue (specifically the mantle) of a living shelled mollusk. Just like the shell of a mollusk, a pearl is made up of calcium carbonate in minute crystalline form, which has been deposited in concentric layers. The ideal pearl is perfectly round and smooth, but many other shapes of pearls occur. The finest quality natural pearls have been highly valued as gemstones and objects of beauty for many centuries, and because of this, the word pearl has become a metaphor for something very rare, very fine, very admirable, and very valuable.

Valuable pearls occur in the wild, but they are very rare. Cultured or farmed pearls make up the majority of those that are currently sold. Pearls from the sea are valued more highly than freshwater pearls. Imitation or fake pearls are also widely sold in inexpensive jewelry, but the quality of their iridescence is usually very poor - and generally speaking, artificial pearls are easily distinguished from genuine pearls. Pearls have been harvested and cultivated primarily for use in jewellery, but in the past they were also stitched onto lavish clothing. Pearls have also been crushed and used in cosmetics, medicines, or in paint formulations.

Pearls that are considered to be of gemstone quality are almost always nacreous and iridescent, like the interior of the shell that produces them. However, most species of shelled mollusks are capable of producing calcareous concretions, creating pearls of lesser shine or less spherical shape. Although these may also be legitimately referred to as "pearls" under U. S. Federal Trade Commission rules, and are formed in the same way, most of them have no value, except as curios.

2. Definition of a pearl

Almost any shelled mollusk can, by natural processes, produce some kind of "pearl" when an irritating microscopic object becomes trapped within the mollusk's mantle folds, but the great majority of these "pearls" are not valued as gemstones. Nacreous pearls, the best-known and most commercially-significant pearls, are primarily produced by two groups of molluscan bivalves or calms. A nacreous pearl is made from layers of nacre, by the same living process as is used in the secretion of the mother of pearl which lines the shell.

A "natural pearl" is one that forms without any human intervention at all, in the wild, and is very rare. Many hundreds of pearl oysters or pearl mussels have to be gathered and opened, and thus killed, to find even one wild pearl, and for many centuries that was the only way pearls were obtained. This was the main reason why pearls fetched such extraordinary prices in the past. A cultured pearl, Conversely, is one that has been formed with human intervention on a pearl farm. The vast majority of pearls on the market today are cultured pearls.

One family of nacreous pearl bivalves, the pearl oysters, lives in the sea while the other, very different groups of bivalves live in freshwater; these are the river mussels such as the fresh water mussel. Saltwater pearls can grow in several species of marine pearl oysters

in the family Pteriidae. Freshwater pearls grow within certain (but by no means all) species of freshwater mussels in the order Unionida, the families Unionidae and Margaritiferidae.

3. Physical properties

The unique luster of pearls depends upon the reflection, refraction and diffraction of light from the translucent layers. The thinner and more numerous the layers in the pearl, the finer is the luster. The iridiscence that pearls display is caused by the overlapping of successive layers, which breaks up light falling on the surface.

In addition, pearls (especially cultured fresh water pearls) can be dyed yellow, green, blue, brown, pink, purple, or black.

4. Freshwater and saltwater pearls

Figure 1: Shell of one species of freshwater pearl mussel, *Margaritifera margaritifera*

Freshwater and saltwater pearls may sometimes look quite similar, but they come from different sources. Natural freshwater pearls form in various species of freshwater mussels, family Unionidae, which live in lakes, rivers, ponds and other bodies of fresh water. These freshwater pearl mussels occur not only in hotter climates, but also in colder more temperate areas (Figure 1). Saltwater pearls grow within pearl oysters, family Pteriidae, which live in oceans. Saltwater pearl oysters are usually cultivated in protected lagoons or volcanic atolls.
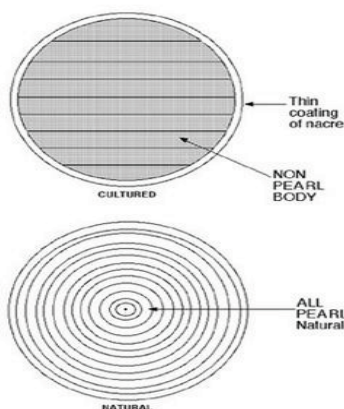
5. Creation of a pearl

Figure 2: Comparison of a cross-section of a cultured pearl, upper, with a natural pearl, lower

The difference between natural and cultured pearls focuses on whether the pearl was created spontaneously by nature - without human intervention- or with human aid. Pearls are formed inside the shell of certain mollusks: as a defense mechanism to a potentially threatening irritant such as a parasite inside its shell, the mollusk creates a pearl to seal off the irritation.

The mantle of the mollusk deposits layers of calcium carbonate ($CaCO_3$) in the form of the mineral aragonite or a mixture of aragonite and calcite (both crystalline forms of calcium carbonate) held together by an organic horn-like compound called conchiolin. The combination of aragonite and conchiolin is called nacre, which makes up mother-of-pearl. The commonly held belief that a grain of sand acts as the irritant is in fact rarely the case. Typical stimuli include organic material, parasites, or even damage that displaces mantle tissue to another part of the animal's body. These small particles or organisms enter the animal when the shell valves are open for feeding or respiration. In cultured pearls, the irritant is typically a cut piece of the mantle epithelium, together with processed shell beads, the combination of which the animal accepts into its body (Figure 2).

6. Natural pearls

Natural pearls are nearly 100% calcium carbonate and conchiolin. It is thought that natural pearls form under a set of accidental conditions when a microscopic intruder or parasite enters a bivalve mollusk, and settles inside the shell. The mollusk, being irritated by the intruder, secretes the calcium carbonate and conchiolin to cover the irritant. This secretion process is repeated many times, thus producing a pearl. Natural pearls come in many shapes, with perfectly round ones being comparatively rare.

6.1 Value of a natural pearl

Quality natural pearls are very rare jewels. The actual value of a natural pearl is determined in the same way as it would be for other "precious" gems. The valuation factors include size, shape, and quality of surface, orient and luster.

Single natural pearls are often sold as a collector's item, or set as centerpieces in unique jewelry. Very few matched strands of natural pearls exist, and those that do often sell for hundreds of thousands of dollars

Keshi pearls, although they often occur by chance, are not considered natural pearls. They are a byproduct of the culturing process, and hence do not happen without human intervention. These pearls are quite small: typically a few millimeters in size. Keshi pearls are produced by many different types of marine mollusks and freshwater mussels in China. Today many "keshi" pearls are actually intentional, with post-harvest shells returned to the water to regenerate a pearl in the existing pearl sac.

6.2 Origin of a natural pearl

Previously, natural pearls were found in many parts of the world. Present day natural pearling is confined mostly to seas of Bahrain. Australia also has one of the world's last remaining fleets of pearl diving ships. Australian pearl divers dive for south sea pearl oysters to be used in the cultured south sea pearl industry. The catch of pearl oysters is similar to the numbers of oysters taken during the natural pearl days. Hence significant numbers of natural pearls are still found in the Australian Indian Ocean waters from wild oysters. X-Ray examination is required to positively verify natural pearls found today.

7. Cultured pearls

Cultured pearls (nucleated and non-nucleated or tissue nucleated cultured pearls) and imitation pearls can be distinguished from natural pearls by X-ray examination. Nucleated cultured pearls are often 'pre-formed' as they tend to follow the shape of the implanted shell bead nucleus. Once the pre-formed beads are inserted into the oyster, it secretes a few layers of nacre around the outside surface of the implant before it is removed after six months or more.

When a nucleated cultured pearl is X-rayed, it reveals a different structure to that of a natural pearl. A cultured pearl shows a solid center with no concentric growth rings, whereas a natural pearl shows a series of concentric growth rings.

7.1 Different types of cultured pearls, including black pearls

Figure 3: A blister pearl, a half-sphere, formed flush against the shell of the pearl oyster

Black pearls, frequently referred to as Black Tahitian Pearls, are highly valued because of their rarity; the culturing process for them dictates a smaller volume output and can never be mass produced. This is due to bad health and/or non-survival of the process, rejection of the nucleus and their sensitivity to changing climatic and ocean conditions. Before the days of cultured pearls, black pearls were rare and highly valued for the simple reason that white pearl oysters rarely produced naturally black pearls, and black pearl oysters rarely produced any natural pearls at all.

Since the development of pearl culture technology, the black pearl oyster found in Tahiti and many other Pacific Island areas has been extensively used for producing cultured pearls. The rarity of the black cultured pearl is now a "comparative" issue. The black cultured pearl is rare when compared to Chinese freshwater cultured pearls, and Japanese and Chinese akoya cultured pearls, and is more valuable than these pearls. However, it is more abundant than the South Sea pearl, which is more valuable than the black cultured pearl. This is simply because the black pearl oyster *Pinctada margaritifera* is far more abundant than the elusive, rare, and larger south sea pearl oyster *Pinctada maxima*, which cannot be found in lagoons, but which must be dived for in a rare number of deep ocean habitats or grown in hatcheries.

Black cultured pearls from the black pearl oyster -*Pinctada margaritifera* - are not South Sea pearls, although they are often mistakenly described as black South Sea pearls. In the absence of an official definition for the pearl from the black oyster, these pearls are usually referred to as "black Tahitian pearls" (Figure 3).

South Sea pearls are the color of their host *Pinctada maxima* oyster, and can be white, silver, pink, gold, cream, and any combination of these basic colors, including overtones of the various colors of the rainbow displayed in the pearl nacre of the oyster shell itself.

8. Development of pearl farming

Today, most pearls used for jewelry are cultured by planting a core or *nucleus* into pearl oysters. The pearls are usually harvested after one year for akoya, 2–4 years for Tahitian and South Sea, and 2–7 years for freshwater. The nucleus is generally a polished

bead made from freshwater mussel shell. Along with a small piece of mantle tissue from another mollusk to serve as a catalyst for the pearl sac, it is surgically implanted into the gonad (reproductive organ) of a saltwater mollusk. In freshwater perliculture, only the piece of tissue is used in most cases, and is inserted into the fleshy mantle of the host mussel. South Sea and Tahitian pearl oysters, also known as *Pinctada maxima* and *Pinctada margaritifera*, which survive the subsequent surgery to remove the finished pearl, are often implanted with a new, larger nucleus as part of the same procedure and then returned to the water for another 2–3 years of growth.

Despite the common misperception, Mikimoto did not discover the process of pearl culture. The accepted process of pearl culture was developed by William Saville-Kent in Australia and brought to Japan by Tokichi Nishikawa and Tatsuhei Mise. Nishikawa was granted the patent in 1916, and married the daughter of Mikimoto. Mikimoto was able to use Nishikawa's technology. After the patent was granted in 1916, the technology was immediately commercially applied to akoya pearl oysters in Japan in 1916. Mise's brother was the first to produce a commercial crop of pearls in the akoya oyster. Mitsubishi's Baron Iwasaki immediately applied the technology to the south sea pearl oyster in 1917 in the Philippines, and later in Buton, and Palau. Mitsubishi was the first to produce a cultured south sea pearl -although it was not until 1928 that the first small commercial crop of pearls was successfully produced.

The original Japanese cultured pearls, known as akoya pearls, are produced by a species of small pearl oyster, *Pinctada fucata martensii*, which is no bigger than 6 to 8 cm in size, hence akoya pearls larger than 10 mm in diameter are extremely rare and highly prized. Today, a hybrid mollusk is used in both Japan and China in the production of akoya pearls. It is a cross between the original Japanese species, and the Chinese species *Pinctada chemnitzii*.

8.1 Pearl Oyster farming

8.1.1 Selection of culture sites

In any farming activity, culture site selection is of paramount importance. Technological and economic considerations play a major role in the selection process. A careful appraisal of the habits of the organism to be cultured would give a resonable level of confidence on the tolerance limits within which the various environmental parameters can vary. Due consideration has to be given to possible effects of fluctuating water flow, primary production, siltation, etc. to obtain the optimum level of growth and production of high quality pearls. Unsuitable levels of environmental factors such as salinity, water temperature, cold water currents and other factors such as red tides, hydrogen sulphide and pollution by industrial and domestic effluents are serious hazards to pearl culture.

Sheltered bays are ideal locations for pearl oyster farms. They offer good protection to the culture structures such as rafts and cages. Shallow coastal waters where the sea is calm most of the year can also be considered as a suitable site.

8.1.2 Environmental conditions

8.1.2.1 Temperature

In temperate regions, the water temperature plays an important role in the biological activities of pearl oysters. In Japan, the optimum temperature for oyster growth has been found to be between 20–25 °C. A temperature below 13 °C causes hybernation. Below 6 °C,

the oysters die. At temperatures above 28 °C, the oysters show exhaustion. The thicknesses of the pearl layers are affected by the minute changes in water temperature during the day and vary considerably according to the season of the year. The deposition of calcium stops at a water temperature of 13 °C. In the Gulf of Kutch, the oysters grow vigorously in winter months when the seawater temperature ranges between 23–27 °C. A slight decrease in temperature triggers spawning in oysters was noticed in the Gulf of Mannar. The growth-temperature relationship is presumably valid only up to a certain temperature for optimum growth.

8.1.2.2 Salinity

Pearl oysters tolerate a wide range of salinity from 24–50 ‰ for a short duration of 2–3 days. Salinities of 14 ‰ and 55 ‰ may cause 100 % mortality among the oysters. The effect of salinity on the growth of pearl oyster has not been clearly investigated. However, it appears that pearl oysters tend to prefer high salinities. Oysters raised in such salinities produce pearls with a golden tint.

8.1.2.3 Bottom

Gravelly bottoms are suitable for pearl oyster farming, while sandy or muddy bottoms should be avoided. Oyster growth is affected by water temperature and nutritional condition of the ground. Repeated culture on the same ground leads to some extent the deterioration of pearl quality. The chemical and physical state of the sea bottom is affected by the organic substances discharged from the oysters and fouling organisms. Periodic removal of such accumulated substances from the bottom of the culture grounds often increases production as well as quality.

8.1.2.4 Depth

The optimum depth for farming pearl oysters is around 15 m. At greater depths, even if the rate of nacre deposition is slower, pearls of high quality with a pinkish colouration are obtained.

8.1.2.5 Silt load

Pearl oysters generally prefer clear waters as high turbidity levels will affect their filtration efficiency. A decline in oyster condition was noted at Veppalodai farm due to the high silt content in the farm area throughout most of the year.

8.1.2.6 Water current

Culture sites should be naturally sheltered against strong winds and waves. Tidal amplitude and currents must be sufficient to allow replenishment of oxygenated water and fresh plankton and flush away waste materials. In strong water currents the formation of the pearl layers is usually fast, but the quality of pearls produced is affected.

8.1.2.7 Primary productivity

The condition of a specific culture ground depends primarily on the chemical constitution of the seawater and on the species and amount of plankton present. Rich nutrients discharged by rivers into the sea are responsible for high primary productivity. The oysters probably derive their chief source of conchiolin from the nitrogen substance of the plankton. The organic matter and calcium dissolved in the seawater are directly absorbed by

the food consumption cells. The calcium passes through the mantle to be deposited on the surface of the shell or pearl in the process of their formation. The presence of trace metals in small quantities influences the colour of the nacre.

8.1.3 Supply of oysters

In pearl oyster farming, oysters collected from the natural beds or reared from naturally collected or cultured spat are used. In the Gulf of Mannar, several pearl banks are distributed off Tuticorn at a distance of 12–15 km and at depths of 12–25 m. Pearl oysters from these beds are collected by skin and SCUBA diving (Figure 4 A). Wide fluctuations in terms of pearl oysters availability have been noted in different pearl banks in this area during the last few centuries as also during the most recent years. In the Gulf of Kutch, the pearl oysters are found on the intertidal flats and the population is sparse. Collection is done by hand.

In Japan, oyster spat are collected by submerging bundles of cedar twigs near the water surface during the peak larval settlement season. Hyzez films and old fish nets are also commonly used as spat collectors. Almost the entire requirement of oyster supply to the pearl culture industry is met by this type of spat collection. Spat collection attempts in India have not been successful, and this may be due to the distance of the pearl oyster beds from coastal waters.

However, India has recently succeeded in producing pearl oyster seed under hatchery conditions, therby providing the industry with a more dependable source of oysters.

8.1.4 Rearing methods

8.1.4.1 Raft culture

Raft culture is considered to be one of the most suitable farming methods in sheltered bays. The size of the rafts can be altered according to the convenience of the situation. A raft of 6×5 m in size can be easily constructed and floated with 4 buoys. Rafts are usually constructed with logs of teak, venteak or casuarina wood, of chosen length with the bottom of about 10 cm diameter tapering to 6 cm diameter at the tip. These logs are arranged according to the requirement and lashed with coir ropes. Floats are attached to the raft to give buoyancy. The floats can be sealed empty diesel drums of 200 l capacity with fibreglass coating, mild steel barrels painted with antisaline/anticorrosive paints or FRP styrofoam floats (Figure IV B and C). Unit raft system is found to be convenient and well suited to the Indian sea conditions. Rafts are moored with anchors at opposite sides with tested quality chains and their direction is decided according to the prevalent wind direction at the specific site (Figure. 4 A-C).

In the long-line culture method, spherical or cylindrical floats which are connected by horizontal synthetic rope or chain are used (Figure 4 D; Fig. 4 D). The oyster cages are suspended from the ropes. This system is good for open sea conditions. In another method of hanging, a hole is drilled near the hinge of the pearl oyster. A small thread is put through the hole, which is then tied to a straw rope coated with tar. The straw ropes are hung from a raft.

8.1.4.2 On-bottom culture

Sea bottoms with a granite or coral stones composition can be used for on-bottom culture. In the Tuticorin Harbour Basin where the breakwater has been constructed with granite stones, the protected portion of the breakwater is used for culturing mother oysters. 1

m of water is available below the low water mark. Due to constant circulation of seawater, settlement of fouling organisms is poor and inconsistent. However, it has been noted that the growth of the mother oyster is slower in on-bottom culture compared to the growth of oysters cultured in raft.

8.1.5 Rearing containers

8.1.5.1 Culture of mother oysters

Box cages, measuring 40×40×15 cm, are used to rear mother pearl oysters. The size of the mesh varies with the size of the oysters to be reared. The frames of the cages are made up of 6 mm mild steel rods, coated with anticorrosive paints or coal tar. Box-cages are useful in general mother oyster culture (Figure 5 A).

To trace the history and performance of individual oysters, frame nets are used. The frames, measuring 60×40 cm each with five compartments, meshed and hinged at one end, open as a book. The oysters are arranged in rows and held in the compartments when closed. The space available in between the two frames is about 10 mm which is sufficient for the oysters to open their valves for feeding and respiration (Figure 5 B).
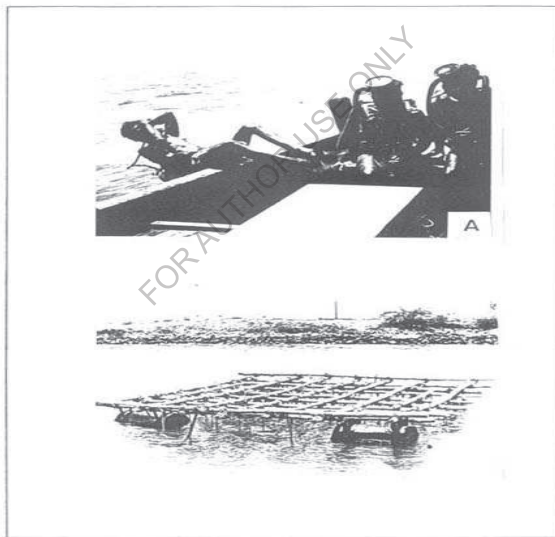

Figure 4 (A) A scuba diver diving to collect pearl oysters and (B) A culture raft floated with mild steel barrels.
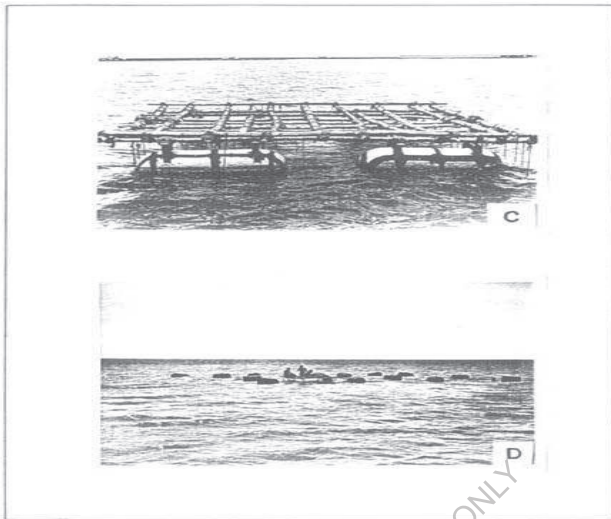
Figure 4. Cont'd. (C) A culture raft with FRP styrofoam buoys and (D) Oyster long-line culture.
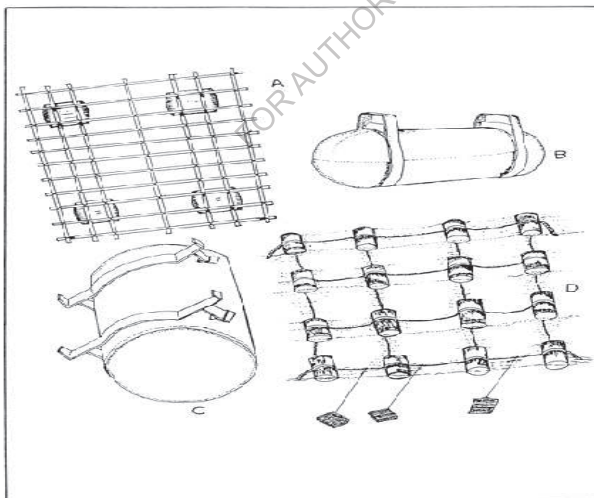

Figure 4 (A) Culture raft constructed with teak poles; (B) A FRP styrofoam buoy; (C) A mild steel buoy, and (D) Oyster long-line culture system.

8.2 Juvenile rearing

Juvenile pearl oysters are reared in netcages (Figure 5 C-D). Synthetic fabrics of velon screen bags whose sides are stretched with a steel rod in the form of a prism are used for rearing of juveniles. The mesh size of the screen depends on the size of juveniles to be reared. The mouth of the bag is tied with a synthetic twine which facilitates opening or closing when required. To provide further protection from predators the bags are placed in old nylon fish net bags. Clogging by silt and by the growth of fouling organisms can be prevented by periodical replacement of the velon screen bag which can be cleaned, sun-dried and reused. Spat of up to 2 cm in size are reared in these small netcages. Box-cages which are used for rearing mother oysters can also be used for juvenile rearing by providing an additional velon screen cover inside the cage.
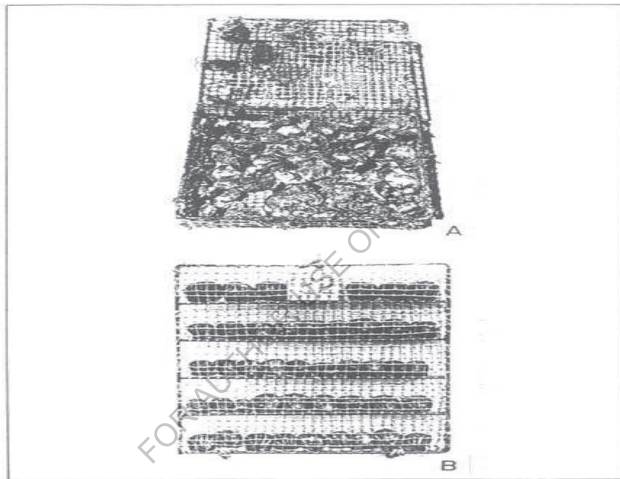

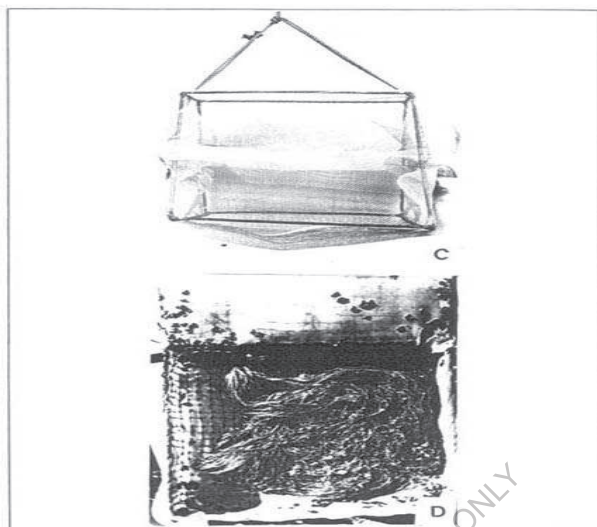**Figure 5** (A) A box-cage containing pearl oysters and (B) A frame netcage with oysters.

Figure 5 Cont'd. (C) A netcage for rearing oyster spat of 3–10 mm in size and (D) Rearing netcage covered with velon screen

9. Pearls and calcareous concretions from other species

Biologically speaking, under the right set of circumstances, almost any shelled mollusk can produce some kind of pearl; however, most of these molluscan pearls have no luster or iridescence. The great majority of mollusk species produces pearls which are not attractive to look at, and are sometimes not even very durable, such that they usually have no value at all, except perhaps to a scientist, a collector, or as a curiosity. These objects used to be referred to as "calcareous concretions" by some gemologists, even though a malacologist would still consider them to be pearls. Valueless pearls of this type are sometimes found in edible mussels, edible oysters, escargot snails, and so on.

A few species produce pearls that can be of interest as gemstones. These species include the bailer shell *Melo*, the giant calm *Tridacna*, various scallop species, Pen shells *Pinna*, and abalones. Another example is the conch pearl (sometimes referred to simply as the 'pink pearl'), which is found very rarely growing between the mantle and the shell of the queen conch or pink conch, *Strombus gigas*, a large sea snail or marine gastropod from the Caribbean sea. These pearls, which are often pink in color, are a by-product of the conch fishing industry, and the best of them display a shimmering optical effect related to chatoyance known as 'flame structure'.

Somewhat similar gastropod pearls, this time more orange in hue, are (again very rarely) found in the horse conch *Pleutoploca gigantea*.
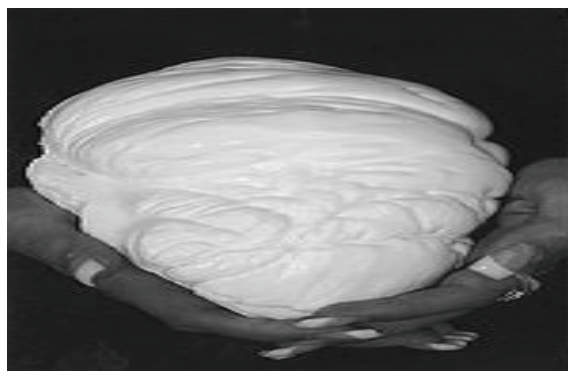
Figure 6: Largest known pearl from a giant clam

The largest pearl known was found in the philippines in 1934. It is a naturally-occurring, non-nacreous, calcareous concretion (pearl) from a giant calm. Because it did not grow in a pearl oyster it is not pearly, but instead it has a porcellaneous surface. In other words, it is glossy like a china plate. Other pearls from giant clams are known to exist, but this is a particularly large one. The pearl weighs 14 lb (6.4 kg) and was supposedly first discovered by an anonymous Filipino Muslim diver off the island of Palawan in 1934. According to the legend as it is currently told, a Palawan chieftain gave the pearl to Wilbur Dowell Cobb in 1936 as a gift for having saved the life of his son. The pearl had been named the "Pearl of Allah" by the Muslim tribal chief, because it resembled a turbaned head. This legend has been discredited, however because this pearl is indeed the product of a giant clam, *Tridacna gigas*, which cannot be grafted (Figure 6). The pearl is also a whole pearl, not a mabe pearl, and whole pearl culturing technology is only 100 years old.

10. Gemological identification

A well equipped gem testing laboratory (e.g., SSEF, Guebelin, GIA, AGTA, HIRCO-INDIA) is able to distinguish natural pearls from cultured pearls by using a gemological x-ray to examine the center of a pearl. With an x-ray it is possible to see the growth rings of the pearl, where the layers of calcium carbonate are separated by thin layers of conchiolin. The differentiation of natural pearls from tissue-nucleated cultured pearls can be very difficult without the use of this x-ray technique.

Natural and cultured pearls can be distinguished from imitation pearls using a microscope. Another method of testing for imitations is to rub the pearl against the surface of a front tooth. Imitation pearls are completely smooth, but natural and cultured pearls are composed of nacre platelets, which feel slightly gritty.

11. Recent pearl production

China has recently overtaken Japan in akoya pearl production. Japan has all but ceased its production of akoya pearls smaller than 8 mm. Japan maintains its status as a pearl processing center, however, and imports the majority of Chinese akoya pearl production. These pearls are then processed (often simply matched and sorted), relabeled as product of Japan, and exported.

In the past couple of decades, cultured pearls have been produced using larger oysters in the south Pacific and Indian Ocean. The largest pearl oyster is the *Pinctada maxima*, which is roughly the size of a dinner plate. South Sea pearls are characterized by their large size and warm luster. Sizes up to 14 mm in diameter are not uncommon. South Sea pearls are primarily produced in Australia, Indonesia and Philippines.

12. Pearls in jewelry

The value of the pearls in jewelry is determined by a combination of the luster, color, size, lack of surface flaw and symmetry that are appropriate for the type of pearl under consideration. Among those attributes, luster is the most important differentiator of pearl quality according to jewelers.

All factors being equal, however, the larger the pearl the more valuable it is. Large, perfectly round pearls are rare and highly valued. Teardrop-shaped pearls are often used in pendants.

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Chapter 21

Sericulture and Seritechnology

1. Sericulture

Sericulture is the rearing of silkworms for the production of raw silk. Although there are several commercial species of silkworms, *Bombyx mori*, is the most widely used and intensively studied. According to Confucian texts, the discovery of silk production by *B. mori* dates to about 2700 BC, although archaeological records point to silk cultivation as early as the Yangshao period (5000 – 10,000 BC). About the first half of the 1st century AD it had reached ancient Khotan, and by AD 300 the practice had been established in India.Later it was introduced to Europe, the Mediterranean and other Asiatic countries. Sericulture has become one of the most important cottage industries in a number of countries like China, the Republic of Korea, Japan, India, Brazil, Russia, Italy and France. Today, China and India are the two main producers, together manufacturing more than 60% of the world production each year.

The technology of sericulture and silk production is well-known. In brief, various aspects involved in it are as follows:

1. **Mulberry Cultivation:** Silkworms feed on mulberry leaves. Hence the rearing of silkworms involves cultivation of mulberry trees, which provide a regular supply of leaves. Worms are introduced through DFLs (Disease Free Layings, i.e., eggs) procured from a quality centre (called grainage). In India, the bulk of mulberry cultivation is done by small farmers (< 4 acres land), usually in clusters of 300-400.
2. **Rearing:** The silkworms are actually larvae of the silkmoth. They are reared in specially made trays in rooms with controlled temperature and humidity and regularly fed mulberry leaves. At a certain stage they convert themselves into cocoons. These cocoons are made from a single filament of material secreted by the pupa and wrapped around itself for protection. These filaments upon hardening constitute silk. On an average, 1 acre of plantation would yield 240 kg of cocoons in an year, starting from 100 DFLs. Depending upon whether it is dryland or irrigated mulberry, farmers can harvest the cocoons 4 to 8 times in an year.
3. **Reeling:** The removal of silk yarn from the cocoons is called reeling. This is done by first cooking them in water to remove the gum, which holds it together, and then unwinding the filaments (reeling). Usually 8-10 cocoons are reeled together. There are three methods for reeling: the charkha, the slightly more advanced cottage basin and the costly automatic machines.
4. **Twisting:** Before weaving, the raw silk is boiled in water to remove remaining gum, dyed and bleached, and then woven into the garment – usually on handloom. In some cases the woven cloth may be dyed and bleached.
5. **Species of Silkworms:** There are four different species of moths, cocoons of which yield differing types of silk:

• **Mulberry Silk** is the most common among them contributing to nearly 95% of world's silk production. It is produced from the cocoons of the moth *Bombyx mori* (Figure 1). Within the species there are many varieties, mainly differentiated according to the number of generations produced annually under natural conditions. Then, hybrids of various kinds have also been developed. Multivoltine varieties

(laying eggs several times a year) have been widely propagated to push up yields, but many feel that they are more vulnerable to pests and hence risky for small farmers. The government provides DFLs of various species through its outlets.
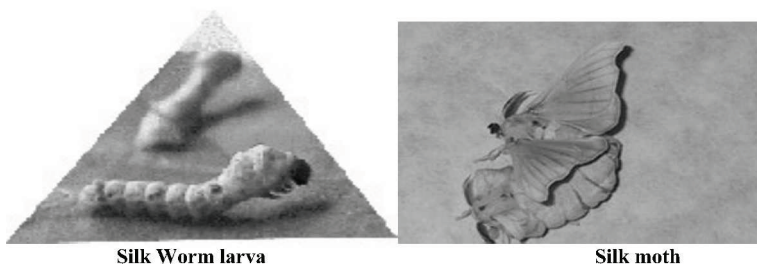

| Silk Worm larva | Silk moth |

Figure 1: *Bombyx mori*

- **Eri** silkworm has two varieties – a wild one and a domesticated one bred on castor leaves. The filament is neither continuous nor uniform. Hence the moths are allowed to emerge before commencing reeling. A white or bright red silk is produced.
- **Tasar** silkworms are wild. The Indian Tasar worm feeds on trees of *Terminalia* species and other minor host plants, while the Japanese and Chinese worms feed on oak and other allied species. Reeling can be done as with mulberry worms.
- **Muga** silkworm is found only in Assam. It feeds on two local species of shrubs – *Machilus bombycina* and *Litsae polyantha*, producing a strong, golden yellow thread.

2. Silkworm Rearing
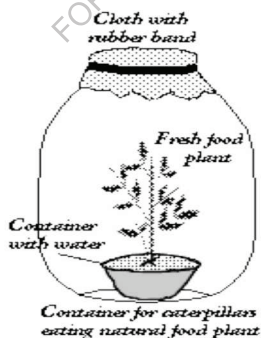### 2.1 Natural food rearing habitat

Figure 2: Container for plant   caterpillars eating natural food

Silkworm larvae are fed cut-up mulberry leaves, and, after the fourth molt, climb a twig placed near them and spin their silken cocoons (Figure 2). The silk is a continuous-filament fiber consisting of fibroin protein, secreted from two salivary glands in the head of each larva, and a gum called sericin, which cements the two filaments together. The sericin is removed by placing the cocoons in hot water, which frees the silk filaments and readies them for reeling. The immersion in hot water also kills the silkworm larvae.

Single filaments are combined to form yarn. This yarn is drawn under tension through several guides and wound onto reels. Finally, the yarn is dried, and the now raw silk is packed according to quality.

Silkworm Needs

Food

The natural food plant of the silkworms is the mulberry tree (*Morus* sp.). Make sure you have a dependable source of pesticide free mulberry leaves before beginning this project. Before feeding the leaves to the larvae, soak the leaves for three minutes in a cleaning solution (three tablespoons of laundry bleach and a drop of dish washing detergent in a gallon of water). Thoroughly rinse the leaves in running water and shake off excess water. Store the leaves in a plastic bag in the refrigerator. The first through third instar larvae require young, tender leaves of mulberry. Offer a leaf in a petri dish and change the leaf daily. After a week you will need to supply more than one leaf. A small piece of damp paper wrapped around the end of the leaf will prolong the freshness. The fourth and fifth instar larvae will need more room and can be set up in the gallon jar with a container to hold mulberry stems and leaves. These older larvae can eat older mulberry leaves. Recut stem ends and quickly insert into water when changing the food plant.

Water

Larvae do not need water if the natural food plant is used because they can obtain all the water they need from the plant.

If you cannot obtain the natural food plant, you must use the artificial diet.
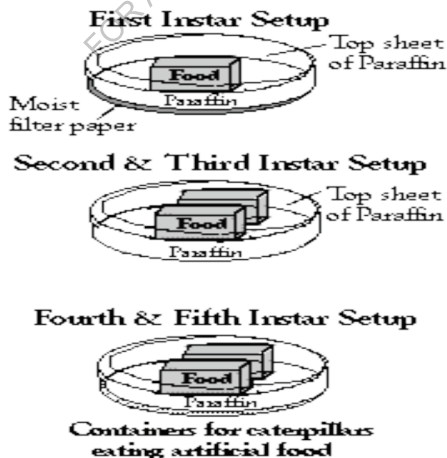
2.2 Arificial Food

Figure 3: Containers for caterpillars eating artificial food

Rearing Habitat

- Disinfected glass petri dishes
- Filter paper or paper toweling
- Spray mister
- Circular sheet of paraffin paper which fits inside the petri dish
- Enough artificial diet for each larva instar
- Alcohol to reduce fungal growth on artificial food.

Food

- Complete instructions should be provided with the purchased artificial food. The following information is gleaned from the Carolina Biological Supply Company informational sheet.
- First instar larva: Dip one bar of diet in water and remove when the water is absorbed. Remove excess water with paper towel. Place a moist piece of filter paper or paper towel in the bottom of the petri dish and cover with a sheet of paraffin paper. Place the moistened diet on the paraffin paper and transfer the larvae. Cover the diet and larva with a sheet of paraffin paper. Check the diet daily and add drops of water to keep it moist.
- Second and third instar larva: After four days, the larva will stop eating and molt. After molting, clean and disinfect the dish or use a new sterile dish. Use the same setup without the moist filter paper. The third instar larva will need at least two petri dishes and will eat at least two bars of diet.
- Fourth and Fifth instar larva: Make sure the larva have enough room and provide additional petri dishes as needed. The top sheet of paraffin paper is no longer needed. Continue to check the diet and keep it moist. If mold appears, remove the larvae and add one or two drops of disinfectant alcohol. Allow the alcohol to evaporate and reassemble setup (Figure 3).

Water
The artificial diet must be moistened with water before the larvae can eat it.

Taking Care

Cleaning

- Frass (droppings) needs to be removed frequently to discourage mold. Gently remove the water container and dump out the frass. Every time you change the food plant, rinse out the container and thoroughly dry it. Periodically wash the water container for the food plant.
- Replace the filter paper and paraffin paper every time you give the larvae new artificial diet. Sterile petri dishes are strongly recommended to reduce fungal growth.

Raising Young

Newly hatched larvae can be transferred by using a small paint brush. Older larvae can be gently picked up with your hands. When the fifth instar larva begins to shrink (after sixth to eighth day), prepare a cocoon nest. Using cellophane paper or paper towels make a roll of paper by twisting one end and placing two or more larvae in it. Twist the other end closed. Store the rolls in the dark at

25°C (77°F). It takes the larva three days to spin the cocoon and then two to three days to molt into a pupa. By cutting the end of the cocoon, a pupa can be gently removed from the cocoon for examination and returned to the cocoon after observation. Although the adults do not fly, the moths have delicate wings that can be damaged by handling.

If you ordered silkworm eggs, the eggs should be placed in a petri dish. After seven days, the eggs will begin to hatch and continue for one to two weeks. Once the first eggs have hatched, check the dish every day for new hatchlings. A day or two before hatching, the egg will darken around the edge. Transfer newly hatched larvae with a fine paint brush to artificial culture or food plant. If adults copulate in captivity, the female will lay eggs within 24 hours. These eggs will not hatch for some time and can be stored.

Precautions

- If you commit to feeding the caterpillar mulberry leaves, make sure you can obtain plenty of leaves. Once the caterpillars have eaten leaves they will not eat the artificial diet.
- Try to maintain the temperature between 68°F to 86°F especially during the first three instars. If you have access to an incubator, keep it at 84°F (29°C). The later instars can be reared with or without an incubator.

Do not place the rearing container where direct sunlight will hit the container. The larvae can easily overheat and die.

3. Production

Stages of production

The stages of production are as follows:

Figure 4: Stages of Silk production

1. The silk moth lays eggs.
2. When the eggs hatch, the caterpillars are fed mulberry leaves.
3. When the silkworms are about 25 days old, they are 10,000 times heavier than when they hatched. They are now ready to spin a silk cocoon.
4. The silk is produced in two glands in the silkworm's head and then forced out in liquid form through openings called spinnerets.
5. The silk solidifies when it comes in contact with the air.
6. The silkworm spins approximately 1 mile of filament and completely encloses itself in a cocoon in about two or three days but due to quality restrictions; the amount of usable silk in each cocoon is small. As a result, 5500 silkworms are required to produce 1 kg of silk.
7. The silkworm then metamorphoses and changes into a moth; however, the silkworm is usually killed with heat before it reaches this stage. The silkworms are killed, because once they reach the moth stage, the moth secretes a fluid to dissolve the silk so it can emerge from the cocoon. This damages the cocoon and the silk then becomes a lower quality. Some silkworms are allowed to live to be used for breeding.
8. The silk is obtained from the undamaged cocoons by brushing the cocoon to find the outside ends of the filament.
9. The silk filaments are then wound on a reel. One cocoon contains approximately 1,000 yards of silk filament. The silk at this stage is known as raw silk. Just one thread consists of 48 individual silk filaments. This could lead to at least 4000 yards in a whole cocoon.
10. A yarn can now be formed by combining several threads of silk.
11. Estimated investments of Rs.12,000 to 15,000 (excluding cost of land and rearing space) is sufficient for undertaking mulberry cultivation and silkworm rearing in one acre of irrigated land.
12. Mulberry takes only six months to grow for commencement of silkworm rearing. Mulberry once planted will go on supporting silkworm rearing year after year for 15–20 years depending on inputs and management provided (Figure 4).

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13. Five crops can be taken in one year under tropical conditions.
14. By adopting stipulated package of practices, a farmer can attain net income levels up to Rs.30000 per acre per annum.

3.1 Cultivation

Figure 5: Cocoon

Silk moths lay eggs on specially prepared paper. The eggs hatch and the caterpillars (silkworms) are fed fresh mulberry leaves. After about 35 days and 4 moltings, the caterpillars are 10,000 times heavier than when hatched, and are ready to begin spinning a cocoon (Figure 5). A straw frame is placed over the tray of caterpillars, and each caterpillar begins spinning a cocoon by moving its head in a "figure 8" pattern. Two glands produce liquid silk and force it through openings in the head called spinnerets. Liquid silk is coated in sericin, a water-soluble protective gum, and solidifies on contact with the air. Within 2–3 days, the caterpillar spins about 1 mile of filament and is completely encased in a cocoon. The silk farmers then kill most caterpillars by heat, leaving some to metamorphose into moths to breed the next generation of caterpillars.

4. Properties

4.1 Physical properties

Silk fibers from the *Bombyx mori* silkworm have a triangular cross section with rounded corners, 5-10 μm wide. The fibroin heavy chain is mostly composed of beta-sheets due to a 59-mer amino acid repeat sequence GAGAGSGAAG[SGAGAG]$_8$Y with some variations. The flat surfaces of the fibrils reflect light at many different angles, giving silk a natural shine. The cross-section from other silkworms can vary in shape and diameter: crescent-like for *Anaphe* and elongated wedge for *tussah*. Silkworm fibers are naturally extruded from two silkworm glands as a pair of primary filaments (brin) which are stuck together, with sericin proteins acting like glue, to form a bave. Bave diameters for tussah silk

can reach 65 μm. Silk has a smooth, soft texture that is not slippery, unlike many synthetic fiber. Its denier is 4.5 g/d when dry and 2.8-4.0 g/d when moist.

Silk is one of the strongest natural fibers but loses up to 20% of its strength when wet. It has a good moisture regain of 11%. Its elasticity is moderate to poor: if elongated even a small amount it remains stretched. It can be weakened if exposed to too much sunlight. It may also be attacked by insects, especially if left dirty.

Silk is a poor conductor of electricity and thus susceptible to static cling.

Unwashed silk chiffon may shrink up to 8% due to a relaxation of the fiber macrostructure. So silk should either be pre-washed before garment construction, or dry cleaned. Dry cleaning may still shrink the chiffon up to 4%. Occasionally, this shrinkage can be reversed by a gentle steaming with a press cloth. There is almost no gradual shrinkage or shrinkage due to molecular-level deformation.

4.2 Chemical properties

Silk is made up of the amino acids Gly-Ser- Gly-Ala and forms Beta pleated sheets. Interchain H-bonds are formed while side chains are above and below the plane of the H-bond network.

The high proportion (50%) of glycine, which is small, allows tight packing and the fibers are strong and resistant to stretching. The tensile strength is due to the many interseeded hydrogen bonds. Since the protein forms a Beta sheet, when stretched the force is applied to these strong bonds and they do not break.

Silk is resistant to most mineral acids but will dissolve in sulfuric acid. It is yellowed by perspiration.

5. Biochemistry of silk worm

Insects mainly belong to two families, viz., Saturnidae and Bombycidae, which spins silk fibre. *Bombyx mori* belongs to Bombycidae produces a delicate twin thread of silk fibroin, which is coated by a protective cover of sericin. Silk protein is a kind of protein like collagen, elastin, keratin, fibroin, sporgin etc., is an essential constituent of cocoon filament.

The silk fibre protein is synthesized by silk gland cells and stored in the lumen of the silk glands. Subsequently, it is converted into silk fibres. When the silkworms secrete the liquid silk during the spinning, it passes through the anterior gland and expelled out through the spinneret opening. Quantity and nature of sericin are fundamental characteristics in conferring distinctive traits to the cocoon.

Sericin is insoluble in cold water; however, it is easily hydrolyzed, where by the long protein molecules brakes down to smaller fractions, which are easily dispersed, or solubilised in hot water. Sericin protein is useful because of its special properties viz., resists oxidation, antibacterial, UV resistant and absorbs and release moisture easily, inhibitory activity of tyrosine and kinase etc. (Figure 6). Sericin, a major component of silk fibre, has been selectively removed from fibroin during the silk manufacturing process to make silk lustrous

and the removed sericin goes as waste material. Now a days, Seri- waste products and Seri-byproducts are used as a value added products.

After Degumming, the leftover is fibroin made up of two brins. Silk fibre can be used for many purposes including textile, medical and industrial applications. The silk fibre is thin, long, light and soft. It is well known for its water absorbency, dyeing affinity, thermo tolerances, insulation properties and luster (Figure 7). It is the raw material for producing precious fabrics, parachutes, tyre lining materials, artificial blood vessels and surgical sutures. Ionic liquid could hold the key to the production of designer silk fibres with enhanced mechanical and optical properties.

The silk fibres have outstanding natural properties, which rival the most advanced synthetic polymers, yet unlike synthetic polymers the production of silk does not require harsh processing conditions. It is reported that the introduction of ionic liquids to silk processing opens an exciting avenue for controlling the microstructure to tune the macroscopic properties.
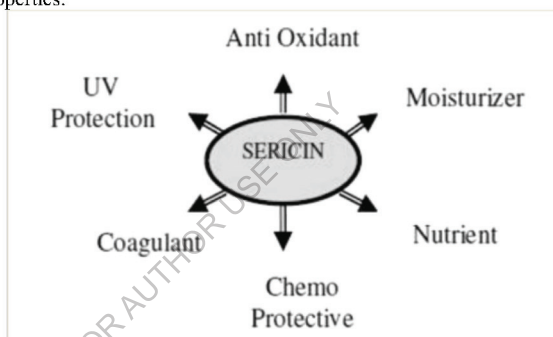

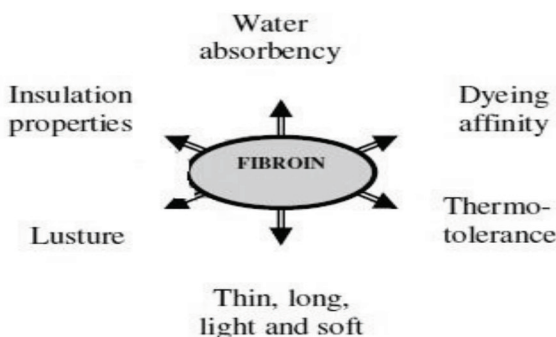**Figure 6. Diagrammatic representation of Attributes of sericin.**

Figure 7. Diagrammatic representation of Attributes of Fibroin.

It is estimated that out of about 1 million tons (fresh weight) of cocoons produced worldwide approximately 4, 00,000 tons of dry cocoon are generated, that have 50,000 tons of recoverable sericin. Indian production of 1,600 tons of silk can be source of about 250 to 300 tons of sericin per year. If this sericin protein is recovered and recycled, it would be a significant economic and social benefit.

5.1 Silk Gland

The natural silk synthesized by the silkworm and spun in the form of a silk cocoon is originally synthesized in the silk gland. Silk gland of *B. mori* is a typical exocrine gland secreting large amount of silk proteins. It is a paired organ consisting of modified labial/salivary glands located at the two lateral sides under the alimentary canal. Each gland is basically a tube made of glandular epithelium with two rows of cells surrounding the lumen.

The cells constituting the gland are huge polyploid cells each with extremely ramified nucleus containing numerous nucleoli. Nuclear ramification develops gradually as the larva grows and reaches conspicuous size in the 4th and 5th instars. Ramification considerably enlarges the nuclear surface and apparently facilitates the transfer of materials related to the silk synthesis between the nucleus and the cytoplasm. According to its morphology and function, the silk gland can be divided into three distinct regions (Figure 8).

The posterior part, about 15 cm long and is composed of about 500 secretary cells, which synthesize silk fibroin. The middle silk gland in the lumen of which silk proteins are stored until spinning, is about 7 cm long and contains about 300 secretory cells producing silk sericin, the protein which cements the fibroin thread of the cocoon. The anterior part about 2 cm long is a thin duct composed of about 250 cells with no known secretory function. *Bombyx mori* silk gland secretes one fibroin and three layers of sericin from the each posterior and middle silk gland in a normal larva.

The Nd-sD mutant is silk fibroin secretion deficient. In the mutant, a disulfide linkage between the heavy (H) chain and light (L) chain is not formed because of partial deletion of the L-chain gene, which is essential for the intercellular transport and secretion of fibroin. The inactivity of the mutant L-chain is the possibility of using the NdsD mutant for the efficient production of recombinants in the silkworm. The posterior silk glands are not sufficiently developed and the liquid fibroin is scarcely secreted, but there is no such disorder in the middle silk glands. A new silkworm race which produces only sericin in Japan in the name of "Sericin Hope" introducing Nd-sD mutant. The analysis of protein variety of middle silk gland cells of the fifth instar larvae of silkworm, *B. mori* at different developmental stages.
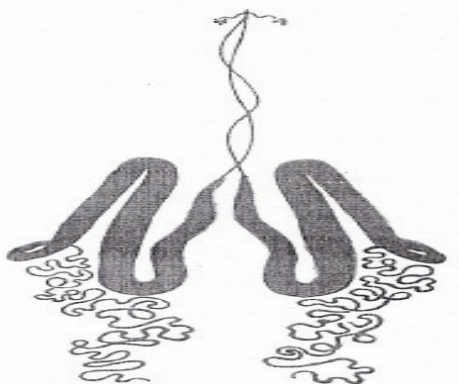
Figure 8. Schematic representation of silk gland of silkworm (*Bombyx mori* L).

The silk gland has the capacity to produce large amount of silk proteins. To use as a foreign proteins, piggyBac vectors were developed to express transgenes in the silk gland using silk gene promoters to drive the expression of the integrated foreign genes. Several promoters were tested to synthesize foreign proteins like procollagen III or globular ones and successfully produced in the silk glands of *Bombyx mori*.

5.2 Composition of the cocoon filament

The silk fibre is almost a pure protein fibre composed of two types of proteins viz., sericin and fibroin. Sericin is chemically a non-filamentous protein. Besides sericin, raw silk also contain other natural impurities namely, fat and waxes, inorganic salts and colouring mater (Table 1). The outer layer of the silk fibre revealed that the sericin content is more in outer layer, where in fibroin content is less (Table 2).

Table 1. Composition of silk in *Bombyx mori*

Component	%
Fibroin	70-80
Sericin	20-30
Wax matter	0.4-0.8
Carbohydrates	1.2-1.6
Inorganic matter	0.7
Pigment	0.2
Total	100

Table 2. Change in cocoon filament of a particular silkworm having dissimilar layers

Layer	Fibroin (%)	Sericin (%)	Ether soaked substance (%)	Ash content (%)
1	64.94	32.41	1.36	1.23
2	74.92	23.15	0.84	1.09
3	78.34	19.79	0.77	1.07
4	79.69	17.86	1.32	1.15
5	79.09	17.78	1.75	1.39

5.3 Silk Protein from the silk gland

Silk fibroin secreted in the lumen of posterior silk gland (PSG) of *B. mori* consists of three protein component: High (H)-chain 350 k Da, Low (L) - chain 26 k Da and Glycoprotein P25 30 k Da. These three types of fibroin (H-chain, L-chain and P 25) are common among different silk producing insects in Lepidoptera, although the fibroin of Saturnidae species secreted as dimer of H chain.

Quantitative enzyme linked immunosorbent assay (ELISA) with specific antibody for each protein component showed that the molar ratio of H-chain, L-chain, and P 25 is 6:6:1 for the fibroin secreted into the posterior silk gland (PSG). The N-linked oligosaccharide chain of P25 has been suggested to be involved in the later interactions. Fibrohexamer was proposed as a functional name of P25 and its central role is maintaining the structure of elementary unit. The disulfide linkage between Cys-172 of the L-chain and Cys-c20 of the H-chain is not responsible for the maintenance of the once formed elementary unit. But the H-L linkage is essential for the large-scale production of fibroin, because fibroin is retained in endoplasmic reticulum in the absence of disulfide linkage between the H and L-chains. The fine structural change of liquid columnar fibroin in the lumen of silk gland was observed during the passage from the posterior to the anterior silk gland during the spinning stage, to analyze the mechanism involved in the formation of a single cocoon filament.

In the posterior part of the posterior silk gland, the columnar fibroin located in the lumen consists of numerous spherical masses of fibroin fibres (MFFs). These MFFs adhered closely together showing a higher concentration in the posterior part of the middle silk gland, and become homogeneous and compact in the anterior silk gland. By the observation of various portion of silk gland, it is concluded that the cocoon filament is composed of oriented elementary fibroin fibres and these fibres are derived from MFFs as they undergo structural change during the passage through the silk gland lumen. Komatsu (1975) postulated molecular aggregating structure of sericin and its changes.

The part of the liquid sericin in the middle silk gland that is easily crystallized by drying is possibly made of amino acid residues with short side-chains and is folded into the globular matrix made of stretches with longer side-chains, crystallizing less readily and on drying, become film of unoriented crystal structure. When this film is swollen in water, stretched and dried, it changes to oriented fibre structure. However, as the orientation is unstable by hot water treatment and thus there is a reversible relationship between the orientation and non-orientation.

The X ray diffraction of fibroin showed 1 mm thick parallel silk fibroin fibres. It is observed that fibroin consists of non- crystalline and crystalline regions. The crystalline region tends to be oriented along the fibre axis because the fibre is drawn as it is extruded from the spinnerets of the silkworm. The crystalline structure and molecular conformation of silk sericin, by X ray diffraction, infrared spectrometry and differential scanning calorimetry of the wild silk (*Antheraea pernyi, Antheraea yamamai, Philosomia cynthia ricini*) by boiling in water. The crystalline structure and molecular conformation of silk sericin obtained from wild silks is not practically different from that of sericin obtained from *B. mori*. Only difference is that the wild silk sericin is relatively insoluble compared to *B. mori*, mainly due to chemical interaction between silk sericin and inorganic minor components or tannins contained originally in wild silk.

The structure of a crystalline form of B. Mori silk fibroin commonly found before the spinning process (known as silk I) has been proposed as a repeated J-turn type II- like structure by combining J obtained from solid-state two dimensional spin-diffusion nuclear magnetic resonance and rotationalecho double resonance.

The molecular and crystal structure of the crystalline modifications of *B. mori*, silk I, is determined by X- ray diffraction method. The cell dimensions are essentially the same as those found in the synthetic model polypeptide (LAla-Gly). The (phi, pI) values of L-Ala and Glee in the repeating unit (-112°,-6°C), (71°, -99°C) which are in the bridge and fourth quadrant regions of the Ramchandran map, respectively.
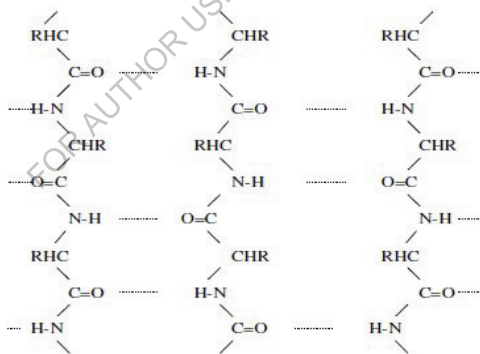

Figure 9. Crystalline structure of the peptide chains in silk fibroin

The observed molecular conformation has a "crank-shaft" or a S- shaped zigzag arrangement, leading to remarkable agreement of observed and calculated structure amplitudes of both dipeptide and hexapeptide sequences, and has a reasonable hydrogen bond networks. X ray structure analyses of the crystalline domains of fibroin show that the peptide chains pack in fully extended forms (Figure 9).

5.4 Molecular confirmation of Sericin and its transition

Sericin extracted from the liquid silk and fresh cocoon shells of a silkworm mutant, which secretes only sericin, is a random coil with 5-10 % β-structure and no α-helix. Sericin, in aqueous solution, displays both random coil and β-structure, but lacks α-helix. Sericins from both the liquid silk in the silk gland and the cocoon filament were studied and confirmed the relationship between solubility in hot water and molecular conformation. To date, most experimental evidences indicate that sericin exists mainly in the random coil or β-structure. It is believed that β structure is intrinsic to liquid silk. Analysis based on circular dichroism spectrum, indicated that sericin extracted from liquid silk for 45 minutes with water contains a small fraction (10%) of α-helix.

During the dissolution of the liquid silk in water, part of the sericin IV become a white suspension due to the coexisting cocoon yarn wax but does not coagulate and the b structure is originally present in the liquid silk. β-structure sericin is more insoluble than random coil sericin. The transition of sericin from its random coil to β- structure takes place by repeated absorption and de-absorption of moisture and by heating during the absorbed state i.e., hygroscopic conditions. Sericins have a cross β-structure when a water solution of sericin containing 50% dioxane or methanol.

Sericin fraction was closest to fibroin in the cocoon filament has molecular chains also arranged in cross β-structure, rather than having the main axis of crystallites oriented at right angle to the fibre axis. The intra- molecular bonds of sericin having random coils are broken by the absorption of water molecules and the folded structure becomes unfolded into an extended structure and is transformed into α, β structure. β-Structure is more stable regarding energy. Part of the sericin thus transformed into β-structure is fixed by its new intermolecular hydrogen bonds and remains crystallized even when the water molecules are removed by drying. In a new cycle of water absorption, the crystalline structure already formed, remains unaffected, whereas, a further fraction of random coils state crystalizes into a b structure there by increasing the portion of β-structure and promotes crystalization. Heating during moisture absorption activates the thermal motions of segments and accelerates transformation. Thus the sericin gets modified in the direction of difficult solubility due to repeated moisture absorption and loss.

5.5 Sericin layer structure or sericin fraction

Scientists have separated sericin of cocoon shell into two proportions: (1) α-sericin and (2) β-sericin. α- Sericin is present in the outer layer of cocoon shell and β-sericin in the inner layer. The α-sericin contains lesser C and H and somewhat more N and O than the β-sericin. The solubility of α-sericin in the boiling water is more than β- sericin. Native sericin is mixture of two substances, sericin A and Sericin B. Sericin in aqueous solutions obtained on degumming silk is not an individual chemical substance but is a mixture of at least two substance, fraction A can be separated by fractional precipitation by adding 15g of ammonium sulphate per 100 ml of sericin solution. When ammonium sulphate is added to the filtrate, sericin B will be precipitated. Some scientists described that on the cocoon thread, the sericin layers are formed from the outside to inside in the order of I, II, III to cover the fibroin. Three fractions of sericin which were different in solubility and called them sericin fractions I, II, and III based on the order of ease of dissolution in hot water and that their ratios were approximately 40:40:20. Based on the different degree of solubility, sericin layers can be called as Sericin A and Sericin B or Sericin α and β or Sericin I, II, and III. The sericin

A is more soluble than the others. It is found that of the sericin I, on the cocoon filament is amorphous where as II and III are crystalline. Four fractions of sericin viz., I, II, III and IV are having different solubilities harder to dissolve being sericin IV. Fourth fraction of sericin was reported to have higher specific gravity and crystallinity than moisture content.

Sericin may be separated into sericin I, II, III and IV by their different solubilities in hot water and assessing the degree of solubility by UV absorption. The greatest sericin content is present in the outer layer of cocoon whereas the least sericin proportion is present in the innermost layer of a cocoon. Fibroin is the principal water insoluble protein (i.e., 78% of the weight of raw silk).

5.6 Amino acid composition in sericin and fibroin

Sericin of mulberry wild silkworm, *Bombyx mori mandarina*, seems to contain the same kind of amino acids as the domesticated silkworm, *B. mori*. However, the wild silkworm sericin contained serine, proline, methoinine, glucosamine, galactosamine and histidine in lower amount and throeonine, glutamic acid, cystine and phenyl amine in higher amount. The content of threonine, galactosamine and glucosamine were significantly higher in the inner layer of cocoons than in the outer layer. Furthermore, the sericin extracted from the floss showed high contents of serine, glycine, valine and tyrosine but low contents in threonine, aspartic acid, alanine, cystine, leucine, glucosamine, galactosamine, lysine and histidine as compared with the sericin of cocoon layer (Table 3).

Table 3. Amino acid composition of sericin obtained from cocoon layer and floss of the mulberry wild silkworm

Amino acid	Cocoon layer			Floss
	Inner	Outer	Whole	
Aspertic acid	18.61	18.3	18.46	10.2
Threonine	11.39	8.44	9.92	6.29
Serine	28.12	29.05	28.58	40.28
Glutamic acid	4.9	4.78	4.84	4.31
Proline	0.51	0.56	0.53	0.66
Glycine	16.9	16.7	16.8	18.17
Alanine	4.84	5.15	5	4.43
Cystine	0.42	0.64	0.53	trace
Valine	2.67	2.91	2.79	3.46
Methionine	0.1	0.11	0.1	0.12
Isoleucine	0.6	0.67	0.63	0.67
Leucine	0.9	1.17	1.03	0.85
Tryrosine	3.28	3.39	3.33	4.09
Phenylamine	0.42	0.47	0.44	0.43
Lysine	2.26	2.89	2.58	1.89
Histidine	0.89	0.99	0.94	0.68
Arginine	3.03	3.41	3.22	3.3

The wild silkworm sericin contained more amino acids with nonpolar side chain and less amino acid with polar side chain than the domesticated silkworm sericin. The co-efficient of pattern-similarity in amino acid composition was high between the sericin of wild silkworm and domesticated silkworms, while sericin of the wild silkworm such as Antheraea or Phylosamia showed significantly low similarity (Table 4). The results show that the amino acid composition of sericin extracted from the cocoon is species specific. Fibroin has high proportions of alanine, glycine and serine. A small amount of cystine residues give a very small amount of sulphar in the fibre. Fibroin contains only a small amount of amino acids, which have acid side chains (Table 5). The isoelectric point of silk fibre is around pH 5. There is low proportion of amino acid residues with large chains in silk.

Table 4. Pattern- similarity coefficient of amino acid composition of the sericin from mulberry wild silkworm (A) and other silkworms (B)

Silkworm	Pattern-similarity coefficient
Bombyx mori mandarina	1
Bombyx mori	0.99
Antherea yamamai	0.97
Antheraea pernyi	0.977
A. paphia mylitta	0.946
A. assamensis	0.974
Philosomia cynthia ricini	0.976

$$\cos\theta = \frac{\sum_{i=1}^{n} A_i.B_i}{\sqrt{\sum_{i=1}^{n} A_{i2}}\sqrt{\sum_{i=1}^{n} B_{i2}}}$$

Pattern-similarity coefficient=S (A, B)=

Table 5. Amino acid composition of silk fibroins (residues/ 1000 residues)

Amino acids	*B. mori* fibre
Glycine	446
Alanine	294
Valine	22
Leucine	5.3
Isoleucine	6.6
Serine	121
Threonine	9.1
Aspartic acid	13
Glutamic acid	10.2
Lysine	3.2
Arginine	4.7
Histidine	1.4

Tyrosine	51.7
Phenylalanine	6.3
Proline	3.6
Tryptophan	1.1
Methionine	1
(Cysteine)^2	2
	Gly>Ala

5.7 SDS- Polyacrylamide gel electrophoresis of Sericin and Fibroin protein

A protein with a low molecular mass of 6027 from the cocoon shell of silkworm, *Bombyx mori* was purified. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) resolved this protein into a single spot with pI 4.3 and Mr 6000. Amino acid sequence analysis revealed that this protein consists of 55 amino acids, six of these being cystine residues and is highly homologous to bovine pancreatic trypsin inhibitor type. The result indicated a significant difference in the protein composition of the same part of middle silk gland (MSG) cells among the second day, the fourth day and the mature silkworm of the fifth instar larvae of silkworm. Eight special protein spots are expressed in anterior part of MSG cells in the second day, sixteen in the fourth day of fifth instar and twenty-four in the mature silkworm.

Nine special protein spots are detected in the middle part of MSG cells in the second day, thirty-three in the fourth day of fifth instar and thirty-four in the mature silkworm. Ten special protein spots are found in posterior part of MSG cell in the second day, seven in the fourth day of fifth instar and twenty-five in the mature silkworm. 2DPAGE patterns of protein from posterior silk gland of 4th day silkworm in 5th instar of different breeds of *B. Mori* were analyzed. Protein forms of posterior silk gland are apparently different for different breeds and different protein spots are found when they compared to middle silk gland of different breed, implying that the transcription, translation of fibroin gene may involve a complex regulation system with great deal of regulation points. The qualitative and quantitative differences in proteins expressed in the middle silk glands of male and female silkworm larvae that differential silk colour are investigated by high resolution twodimensional polyacrylamide gel electrophoresis (2-D PAGE). They reported that there are no distinct differential proteins between the silk gland tissue of female and male larvae within the same variety of sex- limited character.

Isolation of the smallest component of silk out of the series of polymers of the smallest component was detected by polyacrylamide gel electrophoresis, could be converted into the smallest component by reduction and aminoethylation. Fibroin and sericin fraction were separated by precipitation of sericin at pH 5.5. On the gel electrophoresis, sericin showed distinct band but fibroin did not. The component of the fibroin and sericin are fractionated by gel filtration on sepharose 6B. The smallest component in the sericin fraction is purified by re-chromatography and showed a single band on gel electrophoresis. Silk protein solution prepared by solubilization of a whole cocoon with ED/Cu (0.13 M Ethylenediamine/ 0.06 M-cupric hydroxide) solution showed distinct bands on polyacrylamide gel electrophoresis in the presence and absence of SDS, and that there exists a series of polymers.

These polymers are reduced by 2-mercaptoethanol to the smallest component of silk protein, which had an apparent mol wt of 24000. Thus fibroin and sericin can be separated by

precipitation then both the fraction of silk can be examined by polyacrylamide gel electrophoresis. The amino acid compositions of two fractions are shown in Table 6.

Table 6. Amino acid composition of supernatant fraction, the precipitate fraction and the smallest component of silk protein

Proteins	Supernatant Fraction Fibroin	Precipitate Fraction Sericin	Smallest component
Aspartic acid	1.68	12.9	15.2
Threonine	1.17	5.21	3.66
Serine	9.48	18.9	12
Glutamic acid	0.96	4.25	7.5
Proline	0	0.69	2.49
Glycine	46.4	24.2	14.7
Alanine	31.6	15.2	13.2
Valine	2.04	3.34	5.54
Methionine	0	0.11	0
Isoleucine	0.28	1.82	5.5
Leucine	0.22	1.99	5.45
Tryrosine	4.98	4.1	3.7
Phenylamine	0	0.69	2.46
Lysine	0.58	2.07	2.03
Histidine	0.08	0.98	1.45
Arginine	0.29	3.02	3.76
AminoEthylcysteine	0	Trace	0.55

Contents are expressed as mol %

The supernatant fraction known as fibroin is particularly rich in glycine and alanine, which together accounted for 78-mol%. Conversely, the precipitate fraction is known as sericin, which was rich in glycine, serine, alanine and aspertic acid, the combined contents of which amounted to 72-mol%. The results of electrophoresis suggest that components of sericin have define molecular weights but those of fibroin do not.

6. Applications of Sericin and fibroin protein

Silkworm is being used as biofactory for the production of useful protein. Silk proteins are natural polymers and are biodegradable with reactive functional groups that open up possibility to be crosslinked with other polymers to be used in controlled delivery. Like other common biomedical textiles such as polyester, silk contains various polar functional groups that might enhance antibiotic absorption.

Biodegradable materials Environment - friendly biodegradable polymers can be produced by blending sericin with other resins.

The Polyurethane foams incorporating sericin are said to have excellent moisture absorbing and desorbing properties. Polymer films, foams, molding resins, and fibres containing sericin (0.01-50% w/w) can be produce by reacting a composition comprising a polyol, tolylene, di- isocyanet, di-butyltin di-laurate (catalyst) and trichloromonofluoromethane (a blowing agent) in the presence of sericin. The moisture

absorption/desorption rate of the sericin containing polyurethane form is two-to five fold grater than that of control. Other procedures have also been reported for producing sericin-containing polyurethane with excellent mechanical and thermal properties.

Membrane materials

Membrane based separations (e.g., reverse osmosis, dialysis, ultra filtration and micro filtration) are used in process such as desalination of water, production of extremely pure water, the bioprocessing industry and some chemical processes. Pure sericin not easily made into membranes, but membranes of sericin cross-linked, blended, or copolymerized with other substance are made readily, because sericin contains large amount of amino acid with neutral polar functional groups. Sericin and fibroin can be used to make membranes for use in separation processes. The insolubilized silk fibroin membrane could be used to separate the mixture of water and alcohol. A cross- linked thin film made of sericin for use as a separating membrane for water and ethanol. Sericin containing membranes are quite hydrophilic. Acrylonitrile used in making certain synthetic polymers can be copolymerized with sericin to prepare a protein containing synthetic polymer film for separating water from organics.

Functional biomaterials

Sericin film located on lay of liquid crystal can uniformly orient the liquid crystal molecules to provide distortion-free high- quality crystal displays. Sericin- coated film is used on the surface of refrigeration equipment because of its antifrosting action. Use of the coated sericin film is an effective antifrosting method that can be widely applied to refrigerators, deep freezers and refrigerated trucks and ships. Moreover use of the coated film on roads and roof can prevent frost damage. Sericin protein can be coated on surfaces of various durable materials to enhance functionality.

Sericin can be used in preparation of art pigments and for surface protection of articles. The material coated the sericin have excellent weather ability, good permeability and do not warp on drying. Sericin blends with water- soluble polymers, especially with polyvinyl alcohol (PVA). A blended hydrogel made of sericin and fibroin and PVA is said to have excellent moisture absorbing and desorbing properties and elasticity. The hydrogel can be used as a soil conditioner and in medical materials and wound dressing. A cross-linked sericin film for enzyme immobilization with glutaraldehyde as the cross- linking agent was prepared. The heat stability, the electro-osmosis resistance and the stability of the immobilized J-glucosidase on the cross-linked sericin film are higher than the free enzyme.

Medical biomaterials

Silkworm silk fibres have been the primary silk- like material used in biomedical applications particularly as sutures. During decades of use, silk fibres have proven to be effective in many clinical applications. At the same time, some biological responses to the protein have raised questions about biocompatibility. A silk fibroin- based wound dressing that could accelerate healing and could be peeled off without damaging the newly formed skin. The non-crystalline fibroin film of the wound dressing had a water content of 3-16% and a thickness of 10- 100 μm. Subsequently, the wound dressing was made with a mixture of both fibroin and sericin.

A membrane composed of sericin and fibroin is an effective substrate for the proliferation of adherent animal cells and can be used as a substitute for collagen. The attachment and growth of animal cells on films made of sericin and fibroin was noticed. Cell attachment and growth were dependent on maintaining a minimum of around 90% sericin in the composite membrane. Film made of sericin and fibroin has excellent oxygen permeability and is similar to human cornea in its functional properties. It hoped that the sericin- fibroin blended film could be used to form article corneas.

A novel mucoadhesive polymer has been prepared by template polymerization of acrylic acid in the presence of silk sericin. Silk protein can be made into a biomaterial with anticoagulant properties, by a sulfonation treatment of sericin and fibroin. The first evidence of antioxidant action of the silk protein by showing that sericin suppressed *in vitro* lipid peroxidation. Furthermore, sericin also found to inhibit tyrosinase activity. These results suggested that sericin is the valuable natural ingredient for food and cosmetics. The biopolymer sericin has a strong affinity to keratin.

Excessive transepidermal water loss (TEWL) is one of the causes of dry skin and skin moisturizers have been used to overcome it. The silk sericin has resemblance with the natural moisturizing factor (NMR). Sericin gel is prepared by using sericin solution with pluronic and carbopol as a stabilizer to prevent water loss from the upper layer of the skin. It forms a moisturizing, semi-occlusive, protective, antiwrinkle film on the skin surface imparting an immediate, long lasting, smooth, silky feeling. The configuration of sericin is very close to the one of human beings. That is why sericin can naturally saturate into skin and revitalize cells. It is discovered that sericin can restrain the functions of active-oxygen (major factor of aging), which brings wrinkles and dark spots.

The use of oxygen-permeable membranes from silk fibroin and silk sericin, containing about 60% water for contact lens, artificial skin, etc. The other uses of sericin includes, as a soil conditioner, coagulant for purification of waste waters, hygroscopic moisture-releasing polyurethane foams and their manufacture for furniture and interior materials, as additives for health foods to prevent colon cancers, medical composites of sericin, additives to rice cooking, fabric care compositions, light and sunscreen compositions, foam-forming aerosol shaving gels, sericin-coated powders for cosmetics, as dermatitis inhibitor, as wound protection film, nail cosmetics, and chewing gums. Fibroin has been explored as a biomedicine for various applications.

Fibroin powder was processed in such a way to retain its natural, optical beauty. A unique property of this silk powder is its ability to hold and release moisture depending on the temperature and humidity of the surroundings. The extremely fine powder (11.30 size) is particularly ideal for applications in pressed powders, blusher, eye makeup, lipstick and nail enamel.

Sericin and fibroin have been recently explored in the field of drug delivery systems. The properties and application of wound protective membrane made by silk fibroin were studied. It is concluded that the fibroin membrane has good wound healing properties. The fibroin hydrogels prepared either by treating a 2% (w/v) silk fibroin aqueous solution at 4 °C temperature or by adding 30% glycerol could be used as scaffolds able to promote in situ bone regeneration. Using fibroin controlled release tablets, gels and mesosphere have been prepared. The applicability of fibroin, a major silk protein, to controlled release type dosage tablets is investigated *in vitro* and *in vivo*. The sulfated silk fibroins have anti-HIV-1 activity

in vitro, apparently due to interference with the adsorption of virus particles to CD4$^+$ cells, and completely blocked virus binding to the cells at a concentration of 100 micro gm/ml.

The silk fibroin can be used as the substratum for the culture of animal cells in place of collagen. The recovery of uranium from dilute aqueous solutions using silk fibroin can be done. The aqueous solution of fibroin is used to prepare a membrane for immobilization of *Aspergillus niger* glucose-oxidase and *Pseudomonas fluorescens* lyophilized cells. The fibroin membrane is used to immobilize coenzed insect cell culture as a vaccine.

The Recombinant human-like collagen (RHLC) is blended with fibroin to prepare a novel biocompatible film as a scaffold material for hepatic tissue engineering applications. Solution blending is used to incorporate RHLC with silk fibroin to enhance the blend films biocompatibility and hydrophilicity while maintaining elasticity. Soluble fibroin enhances Insulin sensitivity and glucose metabolism in 3T3-L1 Adiposities. The fibroin protein is one kind of biological materials used for artificial skin and others medical application. Silk fibroin membrane supports the application as photo sensor for hydrogen peroxide analysis. Silk protein sericin, suppress DMBA-TPA induced mouse skin tumorigenesis by reducing oxidative stress, inflammatory responses and endogenous tumor promoter TNF-alpha.

7. Physical map of *Bombyx mori*

A physical map of the DNA containing the gene for silk fibroin was developed from direct hybridization analysis of restriction endonuclease digests of total *Bombyx mori* DNA using fibroin 125I-mRNA. The orientation of mRNA transcription relative to this map was deduced from the sensitivity of the mRNA coding strand within certain DNA restriction segments to lambda-exonuclease and exonuclease III. The map includes the entire gene coding region (approximately 11×10^6) and large DNA elements which flank the gene at its 5' end (approximately 3×10^6) and 3' end (approximately 6.5×10^6). The coding region is remarkably uniform in its sensitivity to restriction endonucleases. It is completely devoid of sites for most of the enzymes tested, including Hae III, the recognition sequence (d-pG-G C-C) of which might be expected to occur frequently in this large DNA block of 60% G + C content. The fibroin coding region does contain an enormous number of sites for enzymes predicted to have activity from known fibroin mRNA sequences. These results suggest that the fibroin gene core is a large homogeneously repetitive block of DNA with little evidence for sequence divergence, or the presence of qualitatively different sequences, which might create other restriction sensitivities. The map allows a comparison to be made of the fibroin gene "context" in DNA from tissues either active or inactive in fibroin synthesis.

8. Regulation of silk encoding genes

As shown in Figure 10, the silk gland, the specialized salivary glands that produce silk is composed of two secretory parts, the posterior silk gland (PSG) where fibroin is synthesized and the middle silk gland (MSG) that produces the sericins. Fibroin is a three-subunit complex constituted by the heavy chain (H-Fib), the light chain (L-Fib) and fibrohexamerin (formerly referred to as P25). H-Fib is a large protein (4700 amino acid residues) which accounts for 85% of the silk mass. L-Fib is a 26 kDa polypeptide linked to H-Fib by a disulfide bond36. Fibrohexamerin, a 25 kDa peptide interacts with L-Fib/H-Fib complex through hydrophobic interactions. Both small peptides serve as molecular chaperones during the intracellular transport and secretion of the H-chain and are part of the silk thread. Sericins are a family of peptides encoded by two genes *Ser-1* and *Ser-2*, which

glue the silk threads. Previous studies on the regulation of the silk-encoding genes suggest the existence of two intricate levels of transcriptional regulation.

One lies in the existence of silk gland-specific and territory-specific transactivators. The factor SGF1, a forkhead-family protein, belongs to the first category and is responsible for the activation of *Ser-1* in MSG cells and of *fhx* in PSG cells. This factor, present in both territories of the silk gland, likely interacts with specific partners able to discriminate between the posterior and the median subparts.

The second concerns chromatin accessibility. The organization of chromatin around the *fhx* promoter is correlated to the functioning of the gene and thus is supposed to control the access of transactivators to their target DNA. It was shown that the chromatin structure of *fhx* in the PSG (where the gene is expressed) exhibits two DNase hypersensitive sites and a MNase accessible region that are not present in the MSG. This indicates that an open chromatin configuration characterizes the gene in the PSG, whereas it remains non-accessible in the MSG41. This would suggest that during early morphogenesis of the silk gland in the embryo, a mechanism discriminates the *fhx* gene in the two prospective subparts of the gland. The involvement of the same SGF1/forkhead factor at early morphogenesis may suggest that the same factors that regulate transcription in differentiated cells are also involved in this determination process.

Using transgenesis now makes it possible to address new questions on this system. The study of SGF1 expression during silk gland embryonic development will allow elucidation of the role of this factor in morphogenesis, prior to analysing the phenotypes induced by over-expression or inhibition (via RNA interference), or by dominant negative versions of the factor. Transgenesis would also allow the localization of the DNA sequences involved in the recognition of the *fhx* gene to-be-expressed in the future PSG cells, by studying mutated *fhx*-derived transgenes and looking at both their DNA topology and their transcription abilities. It should thus be possible to delineate which sequences are required for defining the tissue specificity of expression in the silk gland.
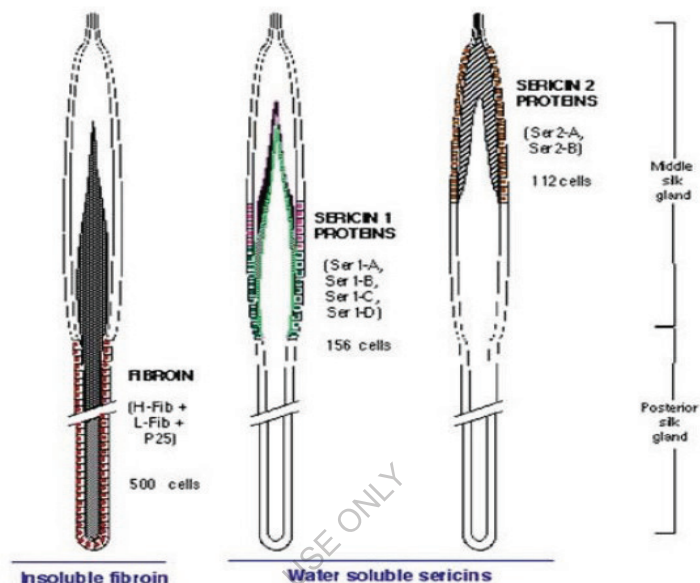
Figure 10. Functional organization of the silk gland. The 500 cells of the posterior silk gland synthesize the three subunits of the fibroin complex, which is secreted into the lumen and accumulates in the middle silk gland where it is covered by successive layers of sericins. The various sericins produced in different subparts of the middle silk gland are encoded by the *Ser-1* and *Ser-2* genes, active in 156 and 112 cells, respectively.

9. Transgenic Silkworm

The productions of useful materials in the transgenic organisms have been developed in plants and mammals. In insects, the method has been requested although the virus systems for the production of recombinant proteins have been established. Since the silkworm possesses the very high ability to synthesize a large amount of silk proteins, it has been expected that very efficient systems for the production of useful materials can be established. In addition, the knowledge about the silkworm science has been extensively accumulated in Japan during these 100 years and the technology handling the silkworm has been developed. Based on these scientific fundamentals, the technology using the transgenic silkworm was thought to have very high potential and it will open important new field of agrobiological sciences.

The recent success of transgenesis of the silkworm *Bombyx mori* opens new prospects on this insect species, exploited both as a powerful biological model system and as a silk producer in industry. In basic research, the examples of *Drosophila melanogaster* and the mouse show how mastered transgenesis can be extremely helpful in understanding basic biological functions related to gene expression and regulation. Similar achievements in the

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silkworm can now be foreseen to decipher basic physiological functions of the insect that, in turn, might sustain further biotechnological and sericultural applications.

The interest in transforming the silkworm rose more than ten years ago, but reaching the objective turned out to be a difficult task. Random integration of DNA following injection of silkworm eggs was attempted years ago in the line of positive results obtained in mouse. This procedure was found non-operative in spite of the possibility to integrate foreign DNA without the help of any vector. This led to an intensive but still unsuccessful search for adapted vectors such as endogenous transposons, pseudo-typed mammalian retroviruses or modified densoviruses. At the same time, *B. mori* genes were expressed in insect cell lines, in somatic cells of embryos, in biolisticated silk glands and in transgenic *D.melanogaster*. Finally the transposon *piggyBac* which had been isolated from the moth *Trichoplusia ni* was found efficient for making transgenic *B. mori* as well as other species (see below) opening the way to transgenesis in a wide spectrum of insects and possibly other Arthropods.

a. The *piggyBac* transposon

piggyBac was first discovered as the responsible agent of the so-called *few polyhedra* (FP) mutations in baculoviruses that were passed through the *T. ni* cell line TN-368. It was subsequently demonstrated that *piggy-Bac*, the insertion of which into the 25 K gene of the virus causes the FP phenotypes, originated from the genome of the insect cells itself. The element is 2.4 kb in length and terminates in 13-bp perfect inverted repeats carrying 5^1CCC…GGG3^1 terminal trinucleotide sequences. An additional internal 19-bp inverted repeat is located asymmetrically with respect to both ends. The transposon sequence can be read as a single ORF encoding a functional transposase with a predicted size of 64 kDa. In the baculovirus genome, inserted elements were systematically found flanked at both ends by the tetranucleotide TTAA, identifying a single chromosome target sequence. Sequences adjacent to the termini of genomic representatives of *piggyBac* were also TTAA, confirming that specificity for the target site is an endogenous property of the transposon. Excision of *piggyBac* from baculovirus insertion sites is precise, regenerating a single TTAA sequence, thereby leaving no footprint behind.

The specificity of the target sequence and the precision of excision implicate the occurrence of a site-specific recombination mechanism and moreover suggest that TTAA is part of the sequence recognized by the *piggy-Bac* transposase. These are remarkable and unique features among type II-transposons. *piggyBac* has only been found in certain strains of *T.ni,* suggesting that it recently invaded the genome of this species. A nearly identical element was found in the genome of the distant dipteran, *Bactrocera dorsalis* while no other tested insect species were shown to harbour *piggyback* elements. Moreover, the experimentally derived vectors are mobile in both species showing that no strong endogenous repression of transposition has developed in the two natural hosts. It is not understood how such a highly mobile element, able to transpose in many diverse species, is not widespread among insects, unless it is a very recent evolutionary event. The lack of host specificity in *piggyBac* mobilization and the absence of established negative control of transposition create a favourable context for the potential occurrence of horizontal gene transfer. As a practical consequence, *piggy-Bac* usage as a transformation vector should be restricted to species that are not aimed at being released in the wild.

b. Transformation methodology in *Bombyx mori*

For transforming *B. mori*, the *piggyBac*-based vectors were derived from the cloned wild type element p3E1.2. As a reporter system, a gene encoding the Green Fluorescent Protein (GFP) was chosen because it provides a neutral dominant marker that is absent in the endogenous genome. The promoter of the cytoplasmic actin gene A3 was used to drive GFP expression as it is active in most of the insect cells and tissues. The non-autonomous helper (pHA3PIG) that expresses the *piggyback* transposase was controlled by the same cytoplasmic actin gene promoter.

The obtained results showed that around 1 to 2 per cent of the injected fertile *G*0 insects give transformed progeny. This efficiency of transformation is within the range obtained in *Drosophila* transgenesis experiments. It means that gene insertion in the germ line is not a highly frequent event. However, the proportion of *G*1 larvae, which from a single brood exhibit a transformed phenotype, varies from less than 1% to more that 25%. This means that the number of transformed gametes in the *G*O animals is highly variable, most probably because integration can occur at different stages of the germ line development.

Genomic DNA analysis showed that the same unique hybridizing fragment is observed in all GFP positive animals, showing that the transgenes are integrated without detectable modifications. The transgenes are stably integrated into the genome and inherited in mendelian proportions over more than 20 generations. In parallel, no extinction of the transgene expression has been observed.

One remarkable property of the system is that in spite of the low frequency of integration, multiple independent insertions occur in the *G*0 transformed parents. The presence of multiple independent inserts in several *G*1 larvae indicates that their *G*0 parent harboured several inserts in a single gamete and that different gametes carried different inserts. However, if most of these inserts were present in the same gonocytes, the distribution of the inserts in the gametes and consequently in the G1 animals would be in favour of a larger number of inserts in each animal. This indicates that prior to meiosis each gonocyte carries only a few transgenes and therefore, that gonocytes are different. This may arise by two distinct mechanisms. One is the late occurrence of integration events during development of the germ-line. Another is the existence of successive rounds of transposition taking place after a single or a limited number of initial insertion events.

Such a hypothesis would explain why, in spite of infrequent insertions in the parental population, the number of inserts is high in the few transformed animals. Successive transpositions could be driven by the stability of the transposase encoded by the helper vector or by the existence of an endogenous cross-mobilizing activity. As the chromosomal insertions are highly stable during the next generations, the second possibility can be ruled out. A similar situation of an unexpected high number of insertions was also observed in transgenic *C. capitata* and it was also attributed to secondary mobilizations after an initial single insertion event. Similar results have been recently obtained in *piggyBac*-transformed *M. domestica* and *B. dorsalis* suggesting that it is a property of the element itself. One can suggest that successive rounds of transposition are not deleterious because of the precise excision of the element. This property makes the system highly efficient and provides the possibility of deriving numerous independent transgenic lines from a few initially transformed individuals.

c. How to improve the transformation procedure?

Silkworm transgenesis is now a routine method leading to a satisfactory yield of transformed animals and to a reliable expression of transgenes during multiple successive generations though the protocol is perfectible and several parameters wait for optimization. It is likely that the site of injection into the egg, the DNA concentration, the helper to selection vector ratio, the DNA delivery procedure could affect gene transfer efficiency. In all probability, the insert size is not strictly limited but this parameter has not been precisely defined and needs further studies.

Three promoters have already been incorporated into *piggyBac* vectors: the transposon promoter itself, the *D.melanogaster* hsp70 promoter and the cytoplasmic actin A3 promoter from *B. mori*. All of them are active in most animal tissues. For further applications tissuespecific promoters are highly desirable. Among many, we are currently assaying the well-characterized silk gland-specific promoters of the silk proteins-encoding genes. For the transposase expressing helper plasmid, the use of a germ line-specific gene promoter active in the very early stages of germ cell precursor multiplication would be very helpful. This will also necessitate basic research on these specialized embryonic cells in *B. mori*.

New selection markers would also be very valuable particularly for inserting different transgenes in the same individual. A recent report described an artificial promoter that was specifically designed to be of universal use. This promoter comprises three binding sites for the *Drosophila* Pax-6 protein, a factor that triggers eye morphogenesis. The conservation of the factor makes this promoter functional in photoreceptor cells of distantly related insect species. Driving the expression of GFP, this promoter proved efficient in the silkworm. Other new markers should be devised and this also will require fundamental studies. The versatility of the system could be also enhanced by constructing *Bombyx* lines that would constitutively produce the transposase in order to remobilize transgenes in already established lines. Secondary mobilization, via appropriate crossings, of inserted *piggyBac* sequences could also be used to detect novel genes by enhancer trapping approaches. Such a transposase-producing line cannot be easily constructed with the *piggyBac* vector itself because it would be highly unstable. Thus, the availability of new vectors remains a desirable goal. The strategy would be to insert the *piggyBac* transposase coding sequence into another vector and to derive a stable line to be crossed with *piggyBac* carrying lines. Such a new vector will also help to produce *Bombyx* lines with multiple inserts. Similarly, we consider the construction of a *Bombyx* line producing the yeast GAL4 protein. This transcriptional activator is produced in a variety of *Drosophila* transgenic lines, where it does not induce any developmental defect. Placing the DNA target sequence recognized by GAL4 in front of transgenes of interest will permit their expression after appropriate crossings. On this basis, various developments are conceivable such as studying dominant negative effect of truncated proteins, the effect of overproduction of protein of interest, of identifying anonymous enhancer sequences. The CRE recombinase specifically recognizes the lox sequence. Creating a *Bombyx* line that expresses this enzyme will be helpful to remove foreign sequences placed in between two inverted lox sites. This strategy is largely and successfully used in mouse transformation studies.

The recent report that homologous recombination can be developed in *B. mori* in order to knock out genes is very promising. The method is not currently a routine procedure due to its very low yield, but its improvement would be of prime interest. The field of *B. mori* transgenesis is largely open to improvements and developments that will require new basic

studies on the system. An international collaboration is needed in order to optimize and exploit the potential of this model both in fundamental research and applied fields.

d. Application of new vector using wild type *Bombyx* kynurenine 3-mono oxygenase gene

A visible marker for finding the transgenic silkworms was developed using *Bombyx* kynurenine 3-mono oxygenase gene under the control of cytoplasmic actin gene promoter (A3KMO) and the w-1 mutant strain as a host. The vector (Figure. 11) was used for the construction of transgenic silkworms for the commercial products. When the vector was used, the transgenic silkworms were detected by the presence of light brown colour in the larval integuments. We performed to construct the transgenic strains using the vector and succeeded to make several strains. In addition, there is a relation between the levels of expression of *KMO* and introduced foreign genes. It was thought that the vector is useful for the production of commercial materials because the vector does not contain any fluorescent marker gene; the use of the vector avoids the patent of fluorescent protein gene.
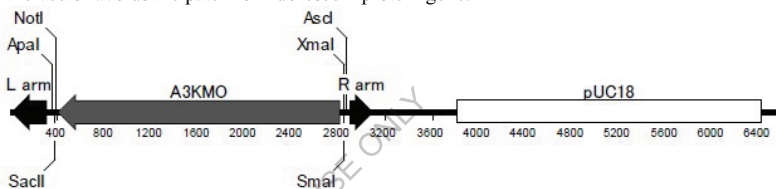

Figure. 11. Physical map of piggyBac vector using the silkworm kynurenine oxigenase gene. A3KMO、marker gene ; arrow、inverted terminal repeat sequence of transposon piggyBac.

e. Identification of Yellow blood gene by use of transgenic silkworms

Yellow blood gene (*Y*) was known to make yellow larval blood and cocoon. Since *Y* is a dominant gene, it may be useful as a new marker. The gene is related to the uptake of carotenoid from mid gut to the blood. Therefore, the carotenoid binding protein (CBP) gene in the mid gut was thought to be the most probable candidate. The carotenoid cDNA from the yellow blood strain was isolated and the cDNA was inserted into the downstream of upstream activation sequence of yeast transcription factor GAL4. This carotenoid gene was introduced into the normal silkworm strain with the normal white blood and cocoon. The expression of the introduced gene was forced in the mid gut and middle part of silk gland by crossing with the GAL4 strain. The blood becomes yellow when it is expressed in the mid gut and that yellow cocoons can be produced when the carotenoid binding protein gene was expressed in mid gut and middle part of silk gland (Figure. 12). From the results it can be concluded Y as the carotenoid-binding protein gene.

Figure. 12 The larvae (left) and cocoons (right) of the transgenic silkworm expressing carotenoid binding protein in the mid gut and middle part of silk gland by GAL4/UAS system. The yellow larva and cocoon are transgenic. The white and opaque larva and white cocoon are non-transgenic as a control.

f. Development of regulation system of the introduced gene in the transgenic silkworms

To develop an efficient expression system for the introduced gene, the effect of different types of GAL4 on the expression of the transgene under the control of UAS were studied. Six different types of the genes were constructed to substitute the sequence of activator domain of GAL4 to that of p65, V16, *Bombyx* REL and RELISH. The activity of the improved GAL4 gene was tested with that of original GAL4 and delta GAL4 in the cultured cells. It was found that the substitution of activator sequence of GAL4 with p65 and V16 is very effective. The GAL4 with p65 activator domain and V16 showed 100 and 10 fold increase of the activity compared to the ordinal GAL4. Studies are in progress to find out how the new GAL4 gene is useful in the transgenic silkworm.

g. Breeding of new silkworm strains for the production of recombinant protein

To breed new silkworm strains that are adapted for the production of recombinant proteins, *Nd-sD* mutant strain was crossed with the Japanese and Chinese parent strains with high productivity. The breeding works very well. The ratios making cocoon and becoming pupa in the bred strains were markedly increased. The breeding was also applied for the transgenic strains with the fluorescent protein gene. The cocoons of the strains with the green fluorescent and red cocoons were much improved. As shown in Figure.13, the size of cocoon is increased and the colour in the cocoon and silks become much clear.
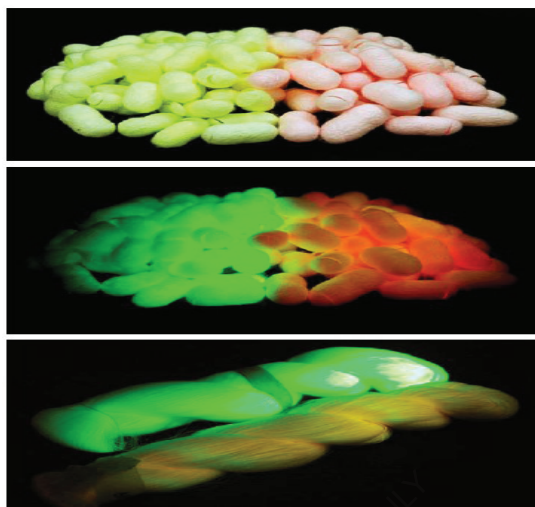
Figure. 13. Cocoons and silk with green and red fluorescence by newly bred transgenic silkworm strains. Upper panel, cocoons observed by visible light; middle panel, cocoons by fluorescent light; lower panel, silks by fluorescent light.

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Chapter 14

In vitro Fertilization

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