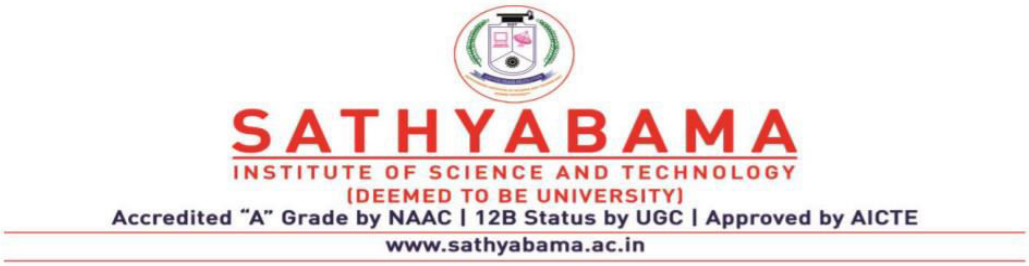


SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – ANIMAL BIOTECHNOLOGY – SBT1305

UNIT I INTRODUCTION TO TISSUE CULTURE

Techniques of cell and tissue Culture - Importance of Aseptic Techniques in cell culture,Environment &Culture media,Serum, Primary culture- Chick Embryo Fibroblast, Chicken,Liver & Kidney culture,Secondary culture,Suspension ,organ culture,Stem cell culture etc., Maintenance & storage of cultures.

TECHNIQUES OF CELL AND TISSUE CULTURE

Tissue culture is the growth of tissues or cells separate from the organism. This is typically facilitated via use of a liquid, semi-solid, or solid growth medium, such as broth or agar. Tissue culture commonly refers to the culture of animal cells and tissues.

The principal purpose of cell, tissue and organ culture is to isolate, at each level of organization, the parts from the whole organism for study in experimentally controlled environments. It is characteristic of intact organisms that a high degree of interrelationship exists and interaction occurs between the component parts. Cultivation *in vitro* places cells beyond the effects of the organism as a whole and of the products of all cells other than those introduced into the culture. Artificial environments may be designed to imitate the natural physiological one, or varied at will by the deliberate introduction of particular variables and stresses.

Virtually all types of cells or aggregates of cells may be studied in culture. Living cells can be examined by cine photomicrography, and by direct, phase-contrast, interference, fluorescence, or ultraviolet microscopy. Fixed cells from culture are suitable for cytological, cytochemical, histological, histochemical, and electron microscopical study. Populations of cells from monolayers or suspension cultures are used for nutritional, biochemical, and immunological work.

Organ culture, the cultivation of whole organs or parts thereof, is particularly suitable for studies of development, of inductive interactions, and of the effects of chemical and physical agents upon the physiological functions of specific organs.

Both cell and organ culture have applications in pathology, *e.g.*, for comparative, developmental, and diagnostic studies of tissues from normal and diseased donors, for

investigations on carcinogenesis, somatic cell genetic variation, viral susceptibility, etc. Cell cultures are widely used in microbiological studies, for investigations of the effects of radiation, and for screening drugs, especially carcinogenic, mutagenic, and radiomimetic agents.

ORGAN CULTURE:

Organ culture is a development from tissue culture methods of research, the organ culture is able to accurately model functions of an organ in various states and conditions by the use of the actual *in vitro* organ itself.

Parts of an organ or a whole organ can be cultured in vitro. The main objective is to maintain the architecture of the tissue and direct it towards normal development. In this technique, it is essential that the tissue is never disrupted or damaged. It thus requires careful handling. The media used for a growing organ culture are generally the same as those used for tissue culture. The techniques for organ culture can be classified into (i) those employing a solid medium and (ii) those employing liquid medium.

Methodology

Embryonic organ culture is an easier alternative to normal organ culture derived from adult animals. The following are four techniques employed for embryonic organ culture.

Plasma clot method

The following are general steps in organ culture on plasma clots.

1. Prepare a plasma clot by mixing 15 drops of plasma with five drops of embryo extract in a watch glass.
2. Place a watch glass on a pad of cotton wool in a petri dish; cotton wool is kept moist to prevent excessive evaporation from the dish.
3. Place a small, carefully dissected piece of tissue on top of the plasma clots in watch glass.

The technique has now been modified, and a raft of lens paper or rayon net is used on which the tissue is placed. Transfer of the tissue can then be achieved by raft easily. Excessive fluid is removed and the net with the tissue placed again on the fresh pool of medium.

Agar gel method

Media solidified with agar are also used for organ culture and these media consist of 7 parts 1% agar in BSS, 3 parts chick embryo extract and 3 parts of horse serum. Defined media with or without serum are also used with agar. The medium with agar provides the mechanical support for organ culture. It does not liquefy. Embryonic organs generally grow well on agar, but adult organ culture will not survive on this medium.

The culture of adult organs or parts from adult animals is more difficult due to their greater requirement of oxygen. A variety of adult organs (e.g. the liver) have been cultured using special media with special apparatus (Towell's II culture chamber). Since serum was found to be toxic, serum-free media were used, and the special apparatus permitted the use of 95% oxygen.

Raft Methods

In this approach the explant is placed onto a raft of lens paper or rayon acetate, which is floated on serum in a watch glass. Rayon acetate rafts are made to float on the serum by treating their 4 corners with silicone.

Similarly, floatability of lens paper is enhanced by treating it with silicone. On each raft, 4 or more explants are usually placed.

In a combination of raft and clot techniques, the explants are first placed on a suitable raft, which is then kept on a plasma clot. This modification makes media changes easy, and prevents the sinking of explants into liquefied plasma.

Grid Method

Initially devised by Trowell in 1954, the grid method utilizes 25 mm x 25 mm pieces of a suitable wire mesh or perforated stainless steel sheet whose edges are bent to form 4 legs of about 4 mm height.

Skeletal tissues are generally placed directly on the grid but softer tissues like glands or skin are first placed on rafts, which are then kept on the grids.

The grids themselves are placed in a culture chamber filled with fluid medium up to the grid; the chamber is supplied with a mixture of O_2 and CO_2 to meet the high O_2 requirements of adult mammalian organs. A modification of the original grid method is widely used to study the growth and differentiation of adult and embryonic tissues.

Limitations

- Results from organ cultures are often not comparable to those from whole animals studies, e.g. in studies on drug action since the drugs are metabolized in vivo but not in vitro.

Applications Of Cell Culture:

1. The mitotic process and its modification by stimulants or suppressors have been studied in many cell types.
2. Visible light has some inhibitory effects upon living cells. The lethal effects of X-

irradiation can be quantified on mouse cells ,and the effects of radiation upon cell constituents and upon DNA and RNA synthesis can be studied.

3. Differentiation at the cellular level has mostly been studied in organ, rather than cell, cultures.
4. The uses of tissue culture in the study of cancer can be studied.
5. Comparison of enzyme activities in cells in culture with those from the mouse have been made.

CELL AND TISSUE CULTURES:

Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions. But in practice it refers to the culturing of cells derived from animal cells.Cell culture was first successfully undertaken by Ross Harrison in 1907. Roux in 1885 for the first time maintained embryonic chick cells in a cell culture. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established.

Primary Culture

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subculture (i.e., passage) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

Cell Line

After the first subculture, the primary culture becomes known as a cell line or sub-clone. Cell lines derived from primary cultures have a limited life span (i.e., they are finite; see below), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

Cell Strain

If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

History

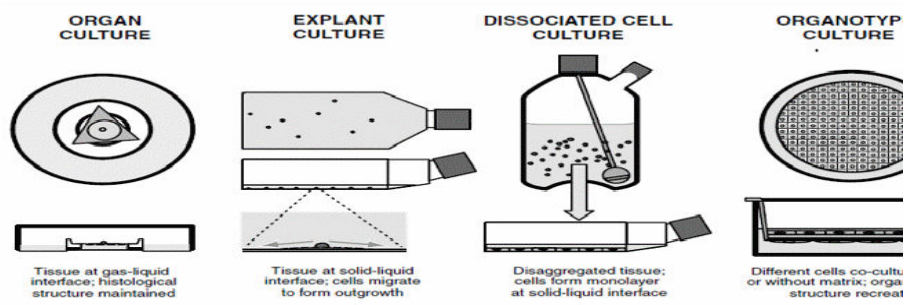
The 19th-century English physiologist Sydney Ringer developed salt solutions containing

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 embryonic 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.

Types of cell culture:

Types of culture. Different modes of culture are represented from left to right. First an organ culture on a filter disk on a triangular stainless steel grid over a well of medium, seen in section in the lower diagram. Second, explant cultures in a flask, with section below and with an enlarged



detail in section in the lowest diagram, showing the explant and radial outgrowth under the arrows. Third, a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram. Fourth, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells.

Morphology of Cells in Culture

Cells in culture can be divided in to three basic categories based on their shape and appearance (i.e., morphology).

1. **Fibroblastic cells**

Fibroblastic (or fibroblast-like) cells are bipolar or multi-polar, have elongated shapes and grow attached to a substrate.

2. **Epithelial-like cells**

Epithelial-like cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.

3. **Lymphoblast-like cells**

Lymphoblast-like cells are spherical in shape and usually grown in suspension without

attaching to a surface.

Cell culture procedures

The following important conditions must be satisfied to achieve successful cell culture:

· Incubationtemperature should be 36°C.

· The pH for growth should be between 7.2 and 7.4.

The levels of glucose and L-glutamine can influence cell growth, and correct levels for each cell line should be checked before attempting to put it into culture (typical levels for glucose and L-glutamine are 1-4 mM and 2 mM, respectively). A range of inorganic ions, amino acids and vitamins are essential for cell survival and will usually be included in basal growth media from proprietary sources. Both oxygen and carbon dioxide are essential and are provided either as a mixture of CO_2 and air supplied to the culture vessel or by sealing the vessel tightly to retain the CO_2 produced by cell metabolism.

Importance of Aseptic technique in cell culture

Skill in aseptic technique is important to maintain sterility during media preparation and cell cultivation procedures. Furthermore, it is a vital component in ensuring operator protection from infectious agents that may be present in culture materials. Some important elements in aseptic technique are:

· Sterilize all glassware for handling cell cultures and media.

· Avoid splashes, spills and aerosols.

· Avoid liquid transfer by pouring.

· When adding (or replacing) medium, never touch the neck of the culture flasks with the bottle containing the medium or use the same pipette to transfer medium to more than one bottle. Ideally, aliquot the total amount of medium required for each batch of culture bottles being handled and store the remainder at 4-8°C. Dedicate separate medium for each cell line.

· Separate clean and contaminated materials in the BSC II.

· Minimize exposure of sterile media and cell cultures to open air (even within the BSC II).

· Perform any final preparation of sterile media (i.e. addition of serum or other additives) before dealing with cell cultures.

Because of the risks of contamination and cross-infection, cell culture in the virus diagnostic laboratory is best carried out in closed vessels, usually screw-capped tubes and flat-

sided bottles. WHO does not recommend the use of 24-well plates for the isolation of polioviruses from stool specimens as this method is inappropriate to conditions encountered in many laboratories of the Global Polio Laboratory Network. Cultures are initially set up in growth medium supplemented with 10% serum. Once the cells have formed a confluent monolayer, cultures are changed to maintenance medium which is designed to maintain cultures in a healthy state for as long as possible without stimulating growth; this is achieved by reducing the serum content, usually to 2%.

Preparation of glassware

Due to the difficulty of cleaning and recycling glassware to culture quality, many laboratories have resorted to using disposable cell culture plastic ware. If a laboratory chooses to use glassware, however, it must ensure that all glassware is meticulously cleaned and sterilized so that cell cultures will not be affected by traces of proteinaceous material, detergent, pyrogens, water deposits and other residual materials which may get deposited on the glassware.

Glassware cleaning protocols should be developed along the lines of the following procedures:

· Use care in handling glassware as most breakages occur during the cleaning process.

· Before cleaning, decontaminate glassware by autoclaving or soaking overnight in chlorine solution (0.5%).

· Decontaminate pipettes in a container containing chlorine.

· Rinse all glassware as soon as possible after use.

· Store soiled items in water containing a disinfectant or cleanser to avoid drying and making items harder to clean.

· Use 7-X, DECON or similar detergent for thorough cleaning of all laboratory glassware. These detergents are easily rinsed from glassware without leaving residues. (DO NOT use domestic dishwashing liquid detergent under any circumstances.)

· Clean glass by scrubbing with a brush. Periodically inspect brushes for wear to avoid scratching glass.

· Thoroughly rinse items in tap water, followed by at least 5-7 changes of distilled or deionized water. Even the smallest residual amounts of cleansers, disinfectants or acids can affect the growth of cell cultures.

· Dry glassware on racks or peg boards and inspect after drying. If glassware is hazy, has a film or blotches are evident, then additional cleaning is required before use.

· Sterilize cell culture glassware using a hot air oven at 180°C for three hours to destroy pyrogens. Non-glass components which may not withstand 180°C should be sterilized by alternate methods such as autoclaving, and re-assembled aseptically.

Chromic acid wash: Some heavily soiled glassware may require vigorous methods to clean and traditionally this has required the use of chromic acid (10% potassium dichromate in 25% sulfuric acid). Chromic acid, however, is a hazardous substance, with safety and environmental concerns. There are effective commercially available substitutes to chromic acid which include: Fisher product, Contrad 70 or VWR Scientific products, Chem-Solv, phosphate-free formulations of RBS-35, PCC-54 and Nochromix (also supplied by Fisher).

If chromic acid must be used, follow all normal safety precautions for using concentrated acids and acid solutions. As with any other cleaning process, all cleaning solutions must be completely rinsed from the glassware through copious changes of tap water followed by several changes of distilled water.

1. **Selection of cell culture systems**

Many cell culture systems support the growth of polioviruses and other enteroviruses. Regional reference laboratories (RRL) are advised to obtain cell cultures from the official collections. Requests for these cell lines should be submitted to IVB/VAM, WHO, Geneva.

National poliomyelitis laboratories can in turn apply to their designated RRL for supplies of these cell lines. As soon as possible after the receipt of cell cultures, a cell bank should be established in liquid nitrogen, or if this is not available, in a mechanical freezer at -70°C or lower. Cells stored at -70°C will not remain viable for very long periods and aliquots should be resuscitated every 4-6 months, passaged to build up numbers, and stored again at -70°C.

2. **Preparation of cell culture systems**

Cells should be received with documented evidence for the key characteristics relating to the quality of cell cultures as described above. In handling cell cultures, laboratory personnel must be concerned not only with preventing microbial contamination of the cultures, but also with avoiding contamination of the working environment with cell culture materials. All cultures must be considered potentially hazardous, whether inoculated or un-inoculated. After use all cultures and their fluids should be decontaminated by autoclaving. Cross-contamination between different cell types, especially continuous cell lines, is an ever-present hazard. To avoid this, different cell lines should never be processed at the same time. All working areas should be thoroughly cleaned between the preparations of different cell types.

Cell culture media employed in virology can be divided into two main categories, growth media and maintenance media. Growth media (GM), high in serum content (usually 10%), promote rapid cell growth. After a monolayer has formed and prior to inoculation with virus, the growth medium is removed and replaced with maintenance medium.

Maintenance media (MM), low in serum content (usually 2%), are intended to keep the cell cultures in a steady state of slow cell replication whilst maintaining cell metabolism during the period of viral replication.Fetal calf serum is the serum of choice: it is good for promoting cell growth and it lacks viral inhibitors. If serum from other sources is used, it must be pre-tested for the presence of inhibitors to the viruses being studied. All sera for cell culture use must be inactivated at 56°C for 30 minutes.

Isolation of cells

Cells can be isolated from tissues for ex vivoculture in several ways. Cells can be easily purified from blood; however only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by enzymatic digestionwith enzymes such as collagenase, trypsin or protease, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as explantculture.

Cells that are cultured directly from a subject are known as primary cells. With the exception of some derived from tumours, most primary cell cultures have limited lifespan. After a certain number of population doublings cells undergo the process of senescence and stop dividing, while generally retaining viability. An established or immortalised cell linehas acquired the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. There are numerous well established cell lines representative of particular cell types.

Culture Environment,Media,Serum

Culture Conditions

Culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physicochemical environment (pH,

osmotic pressure, temperature). Most cells are anchorage dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture).

Maintaining cells in culture

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37^0C, 5% $CO_2$for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed. Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum. One complication of these blood-derived ingredients is the potential for contamination of the culture with viruses or prions, particularly in biotechnology medical applications.Current practice is to minimize or eliminate the use of these ingredients wherever possible, but this cannot always be accomplished. Cells can be grown in suspensionor adherentcultures. Some cells naturally live in suspension, without being attached to a surface, such as cells that exist in the bloodstream. Adherent cells require a surface, such as tissue culture plastic, which may be coated with extracellular matrix components to increase adhesion properties and provide other signals needed for growth and differentiation. Most cells derived from solid tissues are adherent.

Cell line cross-contamination

Cell line cross-contamination can be a problem for scientists working with cultured cells. Cells used in experiments have been misidentified or contaminated with another cell lines. To prevent cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the cell line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing.

Manipulation of cultured cells

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues:

- Nutrient depletion in the growth media
- Accumulation of dead cells.
- Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as

contact inhibition or senescence.

- Cell-to-cell contact can stimulate cellular differentiation

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells.Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g. Amphotericin B) can also be added to the growth media. As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium in order to measure nutrient depletion.

Applications of cell culture

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening and development and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

Primary Culture:

Tissue culture was first devised at the beginning of the twentieth century by Harrison, 1907; Carrel, 1912. Itwas used as a method for studying the behavior of animal cells free of systemic variations that might arise *in vivo* both during normal homeostasis and under the stress of an experiment.

Key Events in the Development of Cell and Tissue Culture:

1907	Frog embryo nerve fiber outgrowth in vitro	Harrison, 1907
1916	Trypsinization and subculture of explants	Rous & Jones, 1916
1920s/30s	Subculture of fibroblastic cell lines	Carrel & Ebeling, 1923
1925–1926	Differentiation in vitro in organ culture	Strangeways & Fell, 1925, 1926
1940s	Introduction of use of antibiotics in tissue culture	Keilova, 1948; Cruikshank & Lowbury, 1952
1952–1955	Establishment the first human cell line, HeLa, from a cervical carcinoma,	Gey et al., 1952

1952	Nuclear transplantation	Briggs & King, 1960
1973	DNA transfer, calcium phosphate	Graham & Van der Eb, 1973
1975	Fibroblast growth factor	Gospodarowicz et al., 1975

Tissue Culture:

Tissue Culture is commonly used as a generic term to include organ culture and cell culture.The term *organ culture* will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue *in vivo*. *Cell culture* refers to a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation. The term *histotypic culture* implies that cells have been reaggregated or grown to re-create a three-dimensional structure with tissuelike cell density, e.g., by cultivation at high density in a filter well, perfusion and overgrowth of a monolayer in a flask or dish, reaggregation in suspension over agar or in real or simulated zero gravity, or infiltration of a three-dimensional matrix such as collagen gel. *Organotypic* implies the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined culture with dermal fibroblasts, in an attempt to generate a *tissue equivalent*.

Harrison [1907] chose the frog as his source of tissue, because it was a cold-blooded animal, and consequently, incubation was not required. Furthermore, tissue regeneration is more common in lower vertebrates.

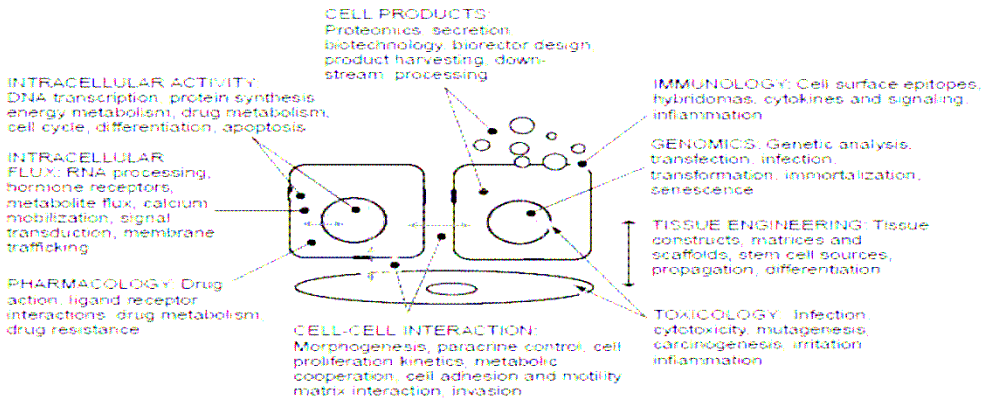


Fig. 1.2. Tissue Culture Applications.

The two major advantages of tissue culture are control of the physiochemical environment (pH, temperature, osmotic pressure, and O_2 and CO_2 tension) and the physiological conditions, which may be kept relatively constant. Most cell lines require supplementation of the medium with serum or other poorly defined constituents. These supplements includes undefined elements such as hormones and other regulatory substances.

Characterization and Homogeneity of Sample

Tissue samples are invariably heterogeneous. Repli-cates—even from one tissue—vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homo-geneous culture of the most vigorous cell type. Hence, at each subculture, replicate samples are identical to each other, and the characteristics of the line may be perpetuated over several generations, or even indefinitely if the cell line is stored in liquid nitrogen.

TABLE 1.2. Advantages of Tissue Culture

Category	Advantages
Physico-chemical environment	Control of pH, temperature, osmolality, dissolved gases
Physiological conditions	Control of hormone and nutrient concentrations
Microenvironment	Regulation of matrix, cell–cell interaction, gaseous diffusion
Cell line homogeneity	Availability of selective media, cloning
Characterization	Cytology and immunostaining are easily performed
Preservation	Can be stored in liquid nitrogen
Validation & accreditation	Origin, history, purity can be authenticated and recorded
Replicates and variability	Quantitation is easy
Reagent saving	Reduced volumes, direct access to cells, lower cost
Control of C × T	Ability to define dose, concentration (C), and time (T)
Mechanization	Available with microtitration and robotics
Reduction of animal use	Cytotoxicity and screening of pharmaceutics, cosmetics, etc.

TABLE 1.3. Limitations of Tissue Culture

Category	Examples
Necessary expertise	Sterile handling Chemical contamination Microbial contamination Cross-contamination
Environmental control	Workplace Incubation, pH control Containment and disposal of biohazards
Quantity and cost	Capital equipment for scale-up Medium, serum Disposable plastics
Genetic instability Phenotypic instability	Heterogeneity, variability Dedifferentiation Adaptation Selective overgrowth
Identification of cell type	Markers not always expressed Histology difficult to recreate and atypical Geometry and microenvironment change cytology

Major Differences *In Vitro* :

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and, as the cells spread out, become mobile, and, in many cases, start to proliferate, so the growth fraction of the cell population increases. When a cell line forms, it may represent only one or two cell types, and many heterotypic cell–cell interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant *in vitro* than *in vivo*, but may not be truly representative of the tissue from which the cells were derived.

Types Of Tissue Culture :

There are three main methods of initiating a culture.,

(1) **Organ culture** implies that the architecture characteristic of the tissue *in vivo* is retained, at least in part, in the culture. Toward this end, the tissue is cultured at the liquid–gas interface (on a raft, grid, or gel), which favors the retention of a spherical or three-dimensional shape.

(2) In **primary explant culture**, a fragment of tissue is placed at a glass (or plastic)–liquid interface, where, after attachment, migration is promoted in the plane of the solid substrate.

(3) ***Cell culture*** implies that the tissue, or outgrowth from the primary explant, is dispersed (mechanically or enzymatically) into a cell suspension, which may then be cultured as an adherent monolayer on a solid substrate or as a suspension in the culture medium.

Because of the retention of cell interactions found in the tissue from which the culture was derived, organ cultures tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue).

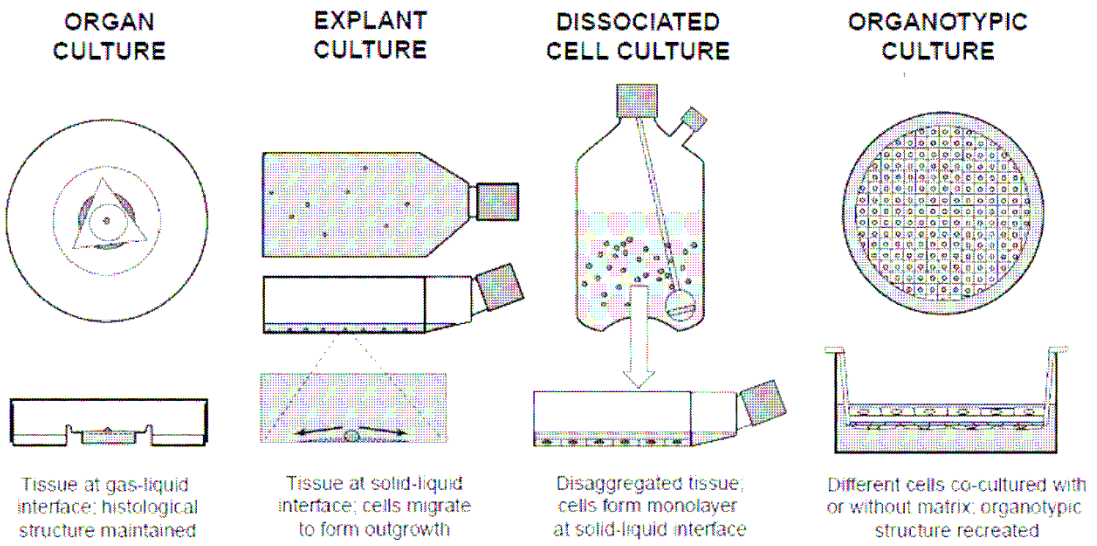


Fig. 1.3. Types of Tissue Culture.

TABLE 1.4. Properties of Different Types of Culture

Category	Organ culture	Explant	Cell culture
Source	Embryonic organs, adult tissue fragments	Tissue fragments	Disaggregated tissue; primary culture, propagated cell line
Effort	High	Moderate	Low
Characterization	Easy; histology	Cytology and markers	Biochemical, molecular, immunological, and cytological assays
Histology	Informative	Difficult	Not applicable
Biochemical differentiation	Possible	Heterogeneous	Lost, but may be reinduced
Propagation	Not possible	Possible from outgrowth	Standard procedure
Replicate sampling, reproducibility, homogeneity	High intersample variation	High intersample variation	Low intersample variation
Quantitation	Difficult	Difficult	Easy; many techniques available

TABLE 1.5. Subculture

Advantages	Disadvantages
Propagation	Trauma of enzymatic or mechanical disaggregation
More cells	Selection of cells adapted to culture
Possibility of cloning	Overgrowth of unspecialized or stromal cells
Increased homogeneity	Genetic instability
Characterization of replicate samples	Loss of differentiated properties (may be inducible)
Frozen storage	Increased risk of misidentification or cross-contamination

TABLE 5.1. Tissue Culture Equipment

Basic requirements
Laminar-flow hood (biohazard if for human cells)
Incubator (humid CO_2 incubator if using open plates or dishes)
5% CO_2 cylinder (for gassing cultures)
Liquid CO_2 cylinders, without siphon (for CO_2 incubator)
Balance
Sterilizer (autoclave, pressure cooker)
Refrigerator
Freezer (for −20°C storage)
Inverted microscope
Soaking bath or sink
Deep washing sink
Pipette cylinder(s)
Pipette washer
Still or water purifier
Bench centrifuge
Liquid N_2 freezer (~35 L, 1,500–3,000 ampoules)
Liquid N_2 storage Dewar (~25 L)
Slow-cooling device for cell freezing (*see* Section 20.3.4)
Magnetic stirrer racks for suspension cultures
Hemocytometer

The Substrate -Attachment And Growth :

The majority of vertebrate cells cultured *in vitro* grow as monolayers on an artificial substrate. Hence the substrate must be correctly charged to allow cell adhesion, or at least to allow the adhesion of cell-derived attachment factors, which will, in turn, allow cell adhesion and spreading.Cells shown to require attachment for growth are said to be *anchorage dependent*; cells that have undergone transformation frequently become *anchorage independent* and can grow in suspension when stirred or held in suspension with semisolid media such as agar.

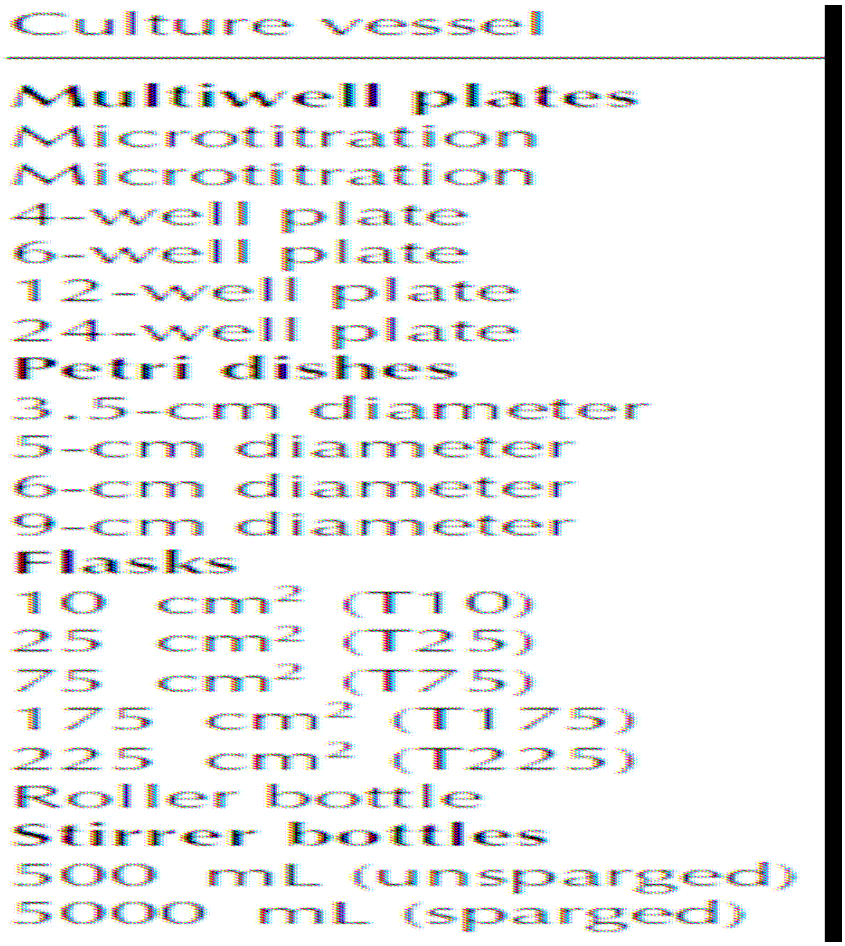
Substrate Materials:

***Glass*.** This was the original substrate because of its optical properties and surface charge, but it has been replaced in most laboratories by synthetic plastic (polystyrene), which has greater consistency and superior optical properties.

***Disposable plastic*.** Single-use sterile polystyrene flasks provide a simple, reproducible substrate for culture. They are usually of good optical quality, and the growth surface is flat, providing uniformly distributed and reproducible monolayer cultures. As manufactured, polystyrene is hydrophobic and does not provide a suitable surface for cell attachment, so tissue culture plastics are treated by corona discharge, gas plasma, or γ -irradiation, or chemically, to produce a charged, wettable surface.

Although polystyrene is by far the most common and cheapest plastic substrate, cells may also be grown on polyvinylchloride (PVC), polycarbonate, polytetrafluorethy-lene (PTFE; Teflon), Melinex, Thermanox (TPX), and a number of other plastics.

Culture vessel characteristics:



Media:

Eagle's Minimal Essential Medium (MEM) [Eagle, 1959] is the commonly used one supplemented with calf, human, or horse serum, protein hydrolysates, and embryo extract. Other cell lines includes L929 cells, HeLa, etc.

Isolation and propagation of cells of a specific lineage may require a selective serum-free medium, whereas cells grown for the formation of products, as hosts for viral propagation, or for non-cell-specific molecular studies rely mainly on Eagle's MEM [Eagle, 1959], Dulbecco's modification of Eagle's medium, DMEM [Dulbecco & Freeman, 1959], or, increasingly, RPMI 1640 [Moore et al., 1967], supplemented with serum. Industrial-scale production techniques now use serum-free media, to facilitate downstream processing and reduce the risk of adventitious infectious agents.

PHYSICOCHEMICAL PROPERTIES :

pH :

Most cell lines grow well at pH 7.4. The optimum pH for cell growth varies relatively little among different cell strains, some normal fibroblast lines perform best at pH 7.4–7.7, and transformed cells may do better at pH 7.0–7.4

Phenol red is commonly used as an indicator. It is red at pH 7.4 and becomes orange at pH 7.0, yellow at pH 6.5, lemon yellow below pH 6.5, more pink at pH 7.6, and purple at pH 7.8.

CO_2 and Bicarbonate :

Carbon dioxide in the gas phase dissolves in the medium, establishes equilibrium with HCO_3^- ions, and lowers the pH.

Buffering :

Culture media must be buffered under two sets of conditions:

(1) open dishes, wherein the evolution of CO_2 causes the pH to rise and

(2) overproduction of CO_2 and lactic acid in transformed cell lines at high cell concentrations, when the pH will fall. A buffer may be incorporated into the medium to stabilize the pH.

Oxygen :

The other major significant constituent of the gas phase is oxygen. Whereas most cells require oxygen for respiration *in vivo*, cultured cells often rely mainly on glycolysis, a high proportion of which, as in transformed cells, may be anaerobic.

Cultures vary in their oxygen requirement, the major distinction lying between organ and cell cultures. Although atmospheric or lower oxygen tensions are preferable for most cell cultures, some organ cultures, particularly from late-stage embryos, new-borns, or adults, require up to 95% O_2 in the gas phase .

Osmolality :

Most cultured cells have a fairly wide tolerance for osmotic pressure .As the osmolality of human plasma is about 290 mosmol/kg, it is reasonable to assume that this level is the optimum for human cells *in vitro*, although it may be different for other species.

Temperature :

The optimal temperature for cell culture is dependent on

(1) the body temperature of the animal from which the cells were obtained,

(2) any anatomic variation in temperature

(3) the incorporation of a safety factor to allow for minor errors in regulating the incubator. Thus the temperature recommended for most human and warm-blooded animal cell lines is 37° C.

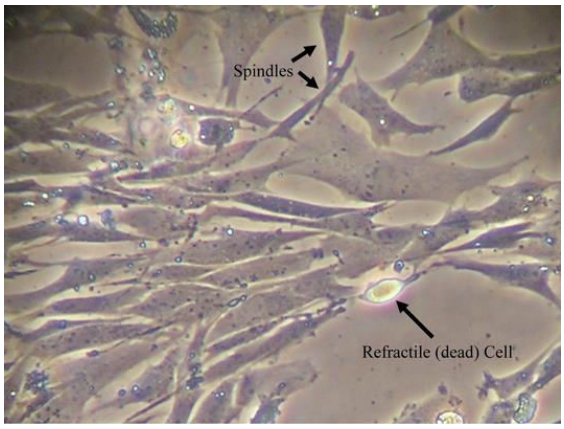
BALANCED SALT SOLUTIONS

A balanced salt solution (BSS) is composed of inorganic salts and may include sodium bicarbonate and, in some cases, glucose.

COMPLETE MEDIA :

The term *complete medium* implies a medium that has had all its constituents and supplements added and is sufficient for the use specified. It is usually made up of a defined medium component, some of the constituents of which, such as glutamine, may be added just before use, and various supplements, such as serum, growth factors, or hormones. Defined media range in complexity from the relatively simple Eagle's MEM [Eagle, 1959], which contains essential amino acids, vitamins, and salts, to complex media such as medium 199 (M199), CMRL 1066, MB 752/1 ,RPMI 1640 ,and F12 and a wide range of serum-free formulations . The complex media contain a larger number of different amino acids, including nonessential amino acids and additional vitamins, and are often supplemented with extra metabolites (e.g., nucleosides, tricarboxylic acid cycle intermediates, and lipids) and minerals.

Primary culture -Chick Embryo Fibroblast



Primary Cell Culture Chick embryo Fibroblast celline

TYPES OF PRIMARY CELL CULTURE :

A primary culture is that stage of the culture after isolation of the cells but before the first subculture. There are four stages to consider: (1) acquisition of the sample, (2) isolation of the tissue, (3) dissection and/or disaggregation, and (4) culture after seeding into the culture vessel. After isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. It appears to be essential for most normal untransformed cells, with the exception of hematopoietic cells, to attach to a flat surface in order to survive and proliferate with maximum efficiency. Transformed cells (*see* Section 18.5.1), on the other hand, particularly cells from transplantable animal tumors, are often able to proliferate in suspension.

The enzymes used most frequently for tissue disag-gregation are crude preparations of trypsin, collagenase, elastase, pronase, dispase, DNase, and hyaluronidase, alone or in various combinations, e.g., elastase and DNase for type II alveolar cell isolation 2002], collagenase with Dispase], and col-lagenase with hyaluronidase [.There are other, nonmammalian enzymes, such as Trypzean ,a recombinant, maize-derived, trypsin, TrypLE (Invitrogen), recombinant microbial, and Accutase and Accumax (Innovative Cell Technologies), also avail-able for primary disaggregation. Crude preparations are often more successful than purified enzyme

preparations, because the former contain other proteases as contaminants, although the latter are generally less toxic and more specific in their action. Trypsin and pronase give the most complete disag-gregation, but may damage the cells. Collagenase and dispase, on the other hand, give incomplete disaggregation, but are less harmful. Hyaluronidase can be used in conjunction with collagenase to digest the intracellular matrix, and DNase is used to disperse DNA released from lysed cells; DNA tends to impair proteolysis and promote reaggregation .

Although each tissue may require a different set of conditions, certain requirements are shared by most of them:

(1) Fat and necrotic tissue are best removed during dissection.

(2) The tissue should be chopped finely with sharp instruments to cause minimum damage.

(3) Enzymes used for disaggregation should be removed subsequently by gentle centrifugation.

(4) The concentration of cells in the primary culture should be much higher than that normally used for subculture, because the proportion of cells from the tissue that survives in primary culture may be quite low.

(5) A rich medium, such as Ham's F12, is preferable to a simple medium, such as Eagle's MEM, and, if serum is required, fetal bovine often gives better survival than does calf or horse. Isolation of specific cell types will probably require selective media (*see* Section 10.2.1 and Chapter 23).

(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

ISOLATION OF THE TISSUE :

Before attempting to work with human or animal tissue, make sure that your work fits within medical ethical rules or current legislation on experimentation with animals (*see* Section 7.9.1). For example, in the United Kingdom, the use of embryos or fetuses beyond 50% gestation or incubation is regulated under the Animal Experiments (Scientific Proce-dures) Act of 1986. Work with human biopsies or fetal mate-rial usually requires the consent of the local ethical committee and the patient and/or his or her relatives .

An attempt should be made to sterilize the site of the resection with 70% alcohol if the site is

likely to be contaminated (e.g., skin). Remove the tissue aseptically and transfer it to the tissue culture laboratory in dissection BSS (DBSS) or transport medium (*see* Appendix I) as soon as possible. Do not dissect animals in the tissue culture laboratory, as the animals may carry microbial contamination. If a delay in transferring the tissue is unavoidable, it can be held at 4° C for up to 72 h, although a better yield will usually result from a quicker transfer.

PRIMARY CULTURE :

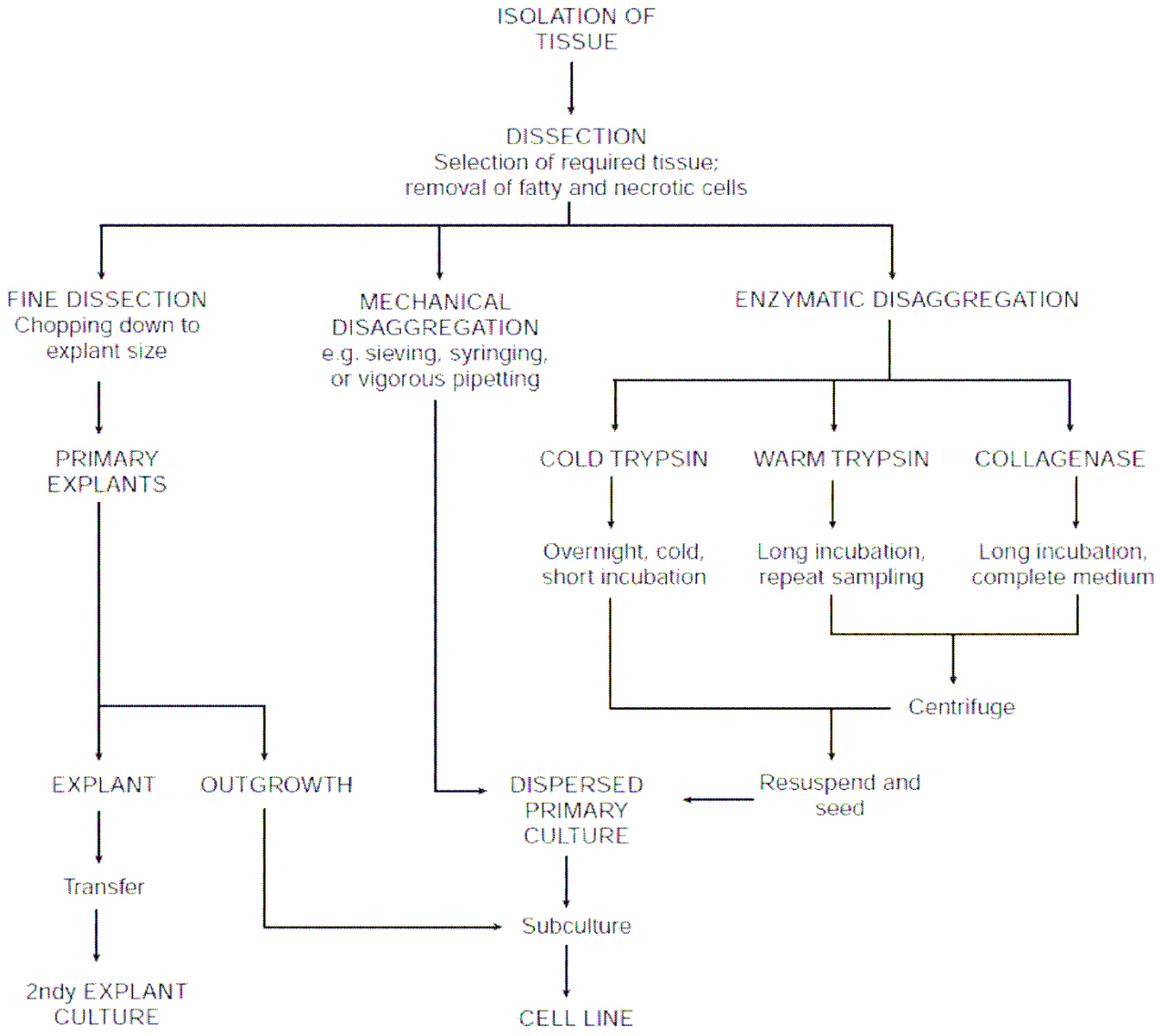
Several techniques have been devised for the disaggregation of tissue isolated for primary culture. These techniques can be divided into

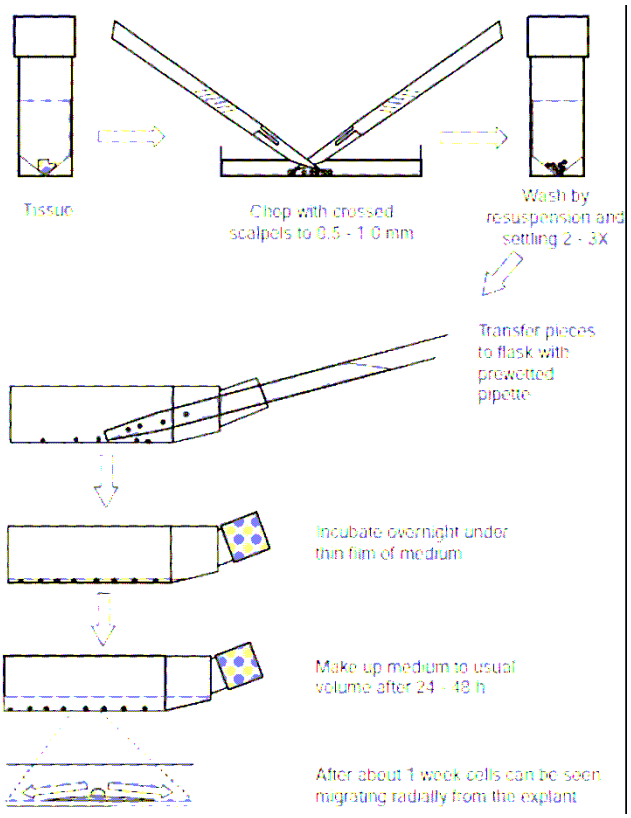
(1) purely mechanical techniques, involving dissection with or without some form of maceration, and

(2) techniques utilizing enzymatic disaggregation (Fig. 12.5). Primary explants are suitable for very small amounts of tissue; enzymatic disaggregation gives a better yield when more tissue is available, and mechanical disaggregation works well with soft tissues, and some firmer tissues when the size of the viable yield is not important.

Primary Explant :

The primary explant technique was the original method developed by Harrison [1907], Carrel [1912], and others for initiating a tissue culture. As originally performed, a fragment of tissue was embedded in blood plasma or lymph, mixed with heterologous serum and embryo extract, and placed on a coverslip that was inverted over a concavity slide. The clotted plasma held the tissue in place, and the explant could be examined with a conventional microscope. The heterologous serum induced clotting of the plasma, and the embryo extract and serum, together with the plasma, supplied nutrients and growth factors and stimulated cell migration from the explant.


ISOLATION OF TISSUE
DISSECTION
Selection of required tissue; removal of fatty and necrotic cells
FINE DISSECTION
Chopping down to explant size
MECHANICAL DISAGGREGATION
e.g. sieving, syringing, or vigorous pipetting
ENZYMATIC DISAGGREGATION
PRIMARY EXPLANTS
COLD TRYPSIN
WARM TRYPSIN
COLLAGENASE
Overnight, cold, short incubation
Long incubation, repeat sampling
Long incubation, complete medium
Centrifuge
EXPLANT
OUTGROWTH
DISPERSED PRIMARY CULTURE
Resuspend and seed
Transfer
Subculture
2ndy EXPLANT CULTURE
CELL LINE



Enzymatic Disaggregation :

Cell–cell adhesion in tissues is mediated by a variety of homotypic interacting glycopeptides (cell adhesion molecules, or CAMs), some of which are calcium dependent (cadherins) and hence are sensitive to chelating agents such as EDTA or EGTA. Integrins, which bind to the arginine-glycine-aspartic acid (RGD) motif in extracellular matrix, also have Ca^{2+}-binding domains and are affected by Ca^{2+} depletion. Intercellular matrix and basement membranes contain other glycoproteins, such as fibronectin and laminin, which are protease sensitive, and proteoglycans, which are less so but can sometimes be degraded by glycanases, such as hyaluronidase or heparinase. The easiest approach is to proceed from a simple disaggregation solution to a more complex solution with trypsin alone or trypsin/EDTA as a

starting point, adding other proteases to improve disaggregation, and deleting trypsin if necessary to increase viability. In general, increasing the purity of an enzyme will give better control and less toxicity with increased specificity but may result in less disaggregation activity.

Mechanical and enzymatic disaggregation of the tissue avoids problems of selection by migration and yields a higher number of cells that are more representative of the whole tissue in a shorter time. However, just as the primary explant technique selects on the basis of cell migration, dissociation techniques will select protease- and mechanical stress-resistant cells. The choice of which trypsin grade to use has always been difficult, as there are two opposing trends: (1) The purer the trypsin, the less toxic it becomes, and the more predictable its action; (2) the cruder the trypsin, the more effective it may be, because of other proteases.

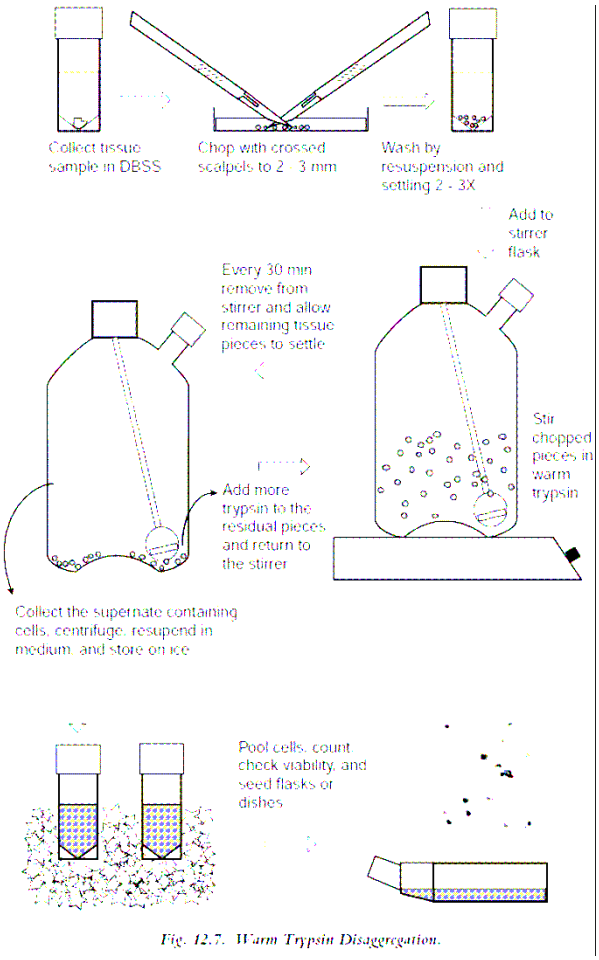
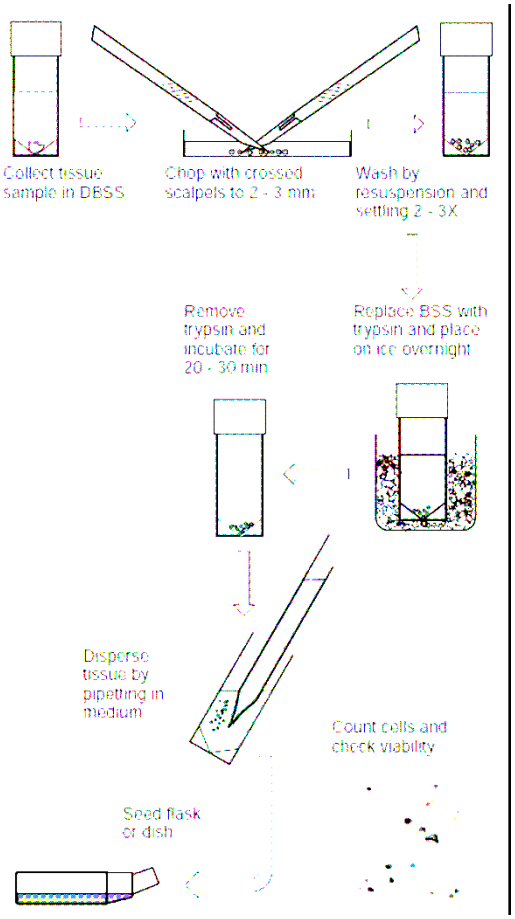
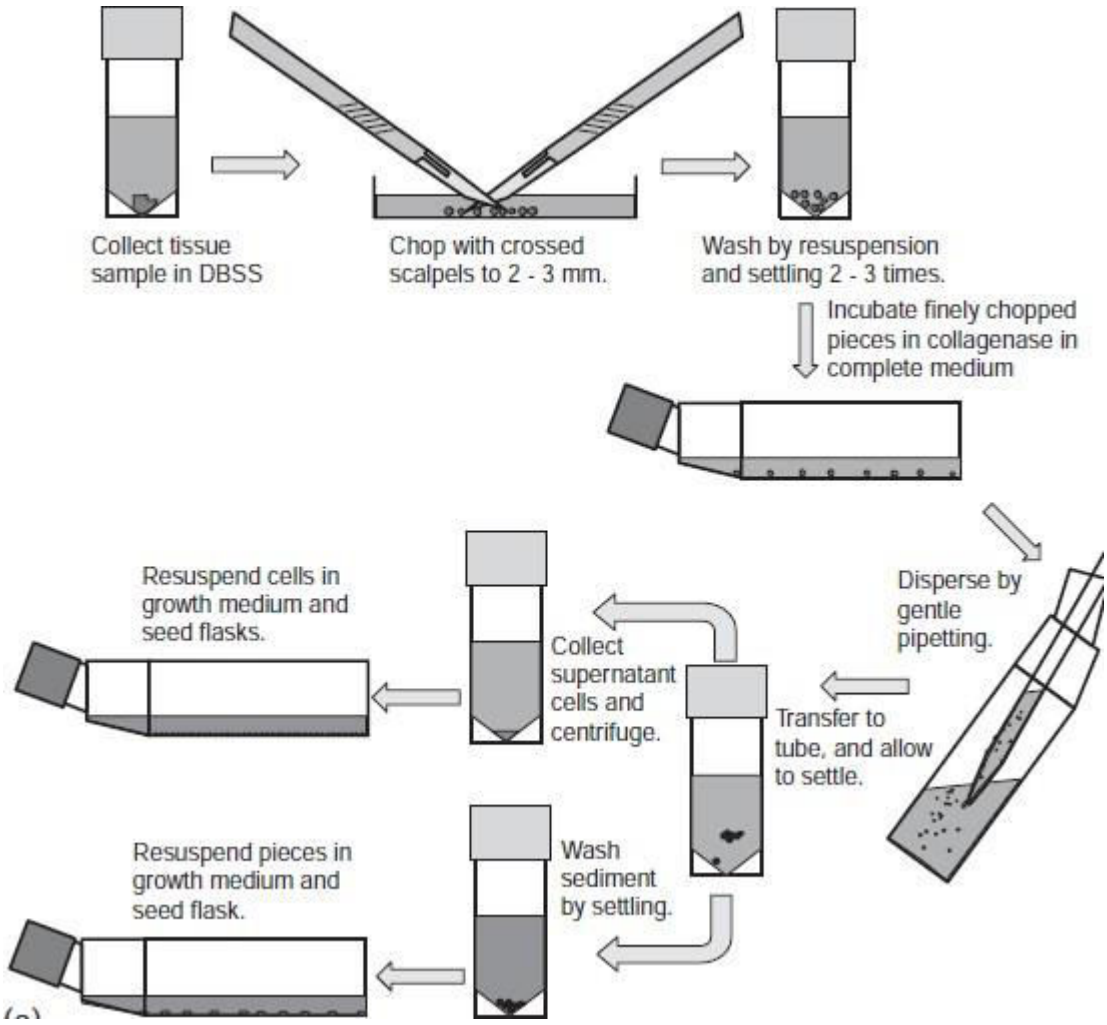


Fig. 12.7. Warm Trypsin Disaggregation.

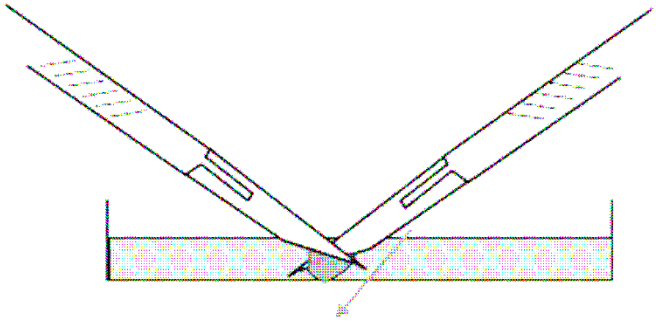


COLD TRYPSIN DISAGGREGATION

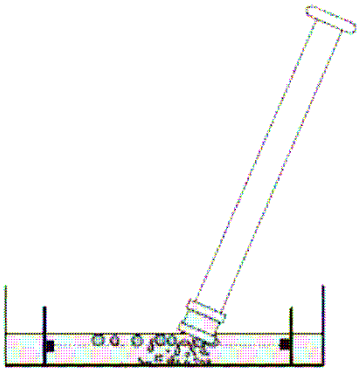


Tissue Disaggregation by Collagenase.

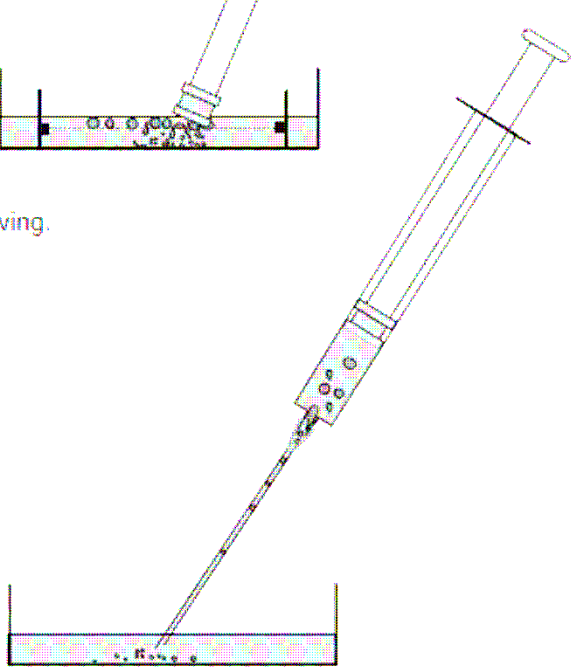
Mechanical Disaggregation. (a) Scraping or ‘‘spillage’’. Cutting action, or abrasion of cut surface, releases cells. (b) Sieving. Forcing tissue through sieve with syringe piston. (Falcon Cell Strainer can be used; (c) Syringing. Drawing tissue into syringe through wide bore needle or canula and expressing.(d) Trituration by pipette. Pippetting tissue fragments up and down through wide bore pipette.



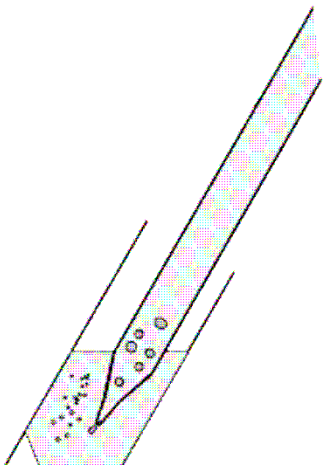
(a) Scraping or "spillage".



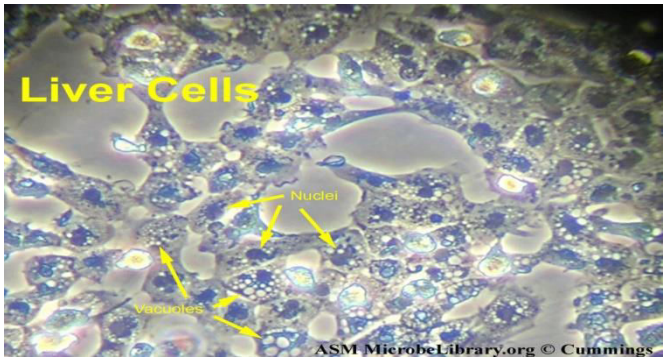
(b) Sieving.



(c) Syringing.



Primary culture -Chicken Liver



This phase-contrast image shows primary chick embryo liver cells grown for 2 weeks in vitro. Note the liver cell nuclei (blue) and numerous cytoplasmic vacuoles containing glycogen.

The liver is the largest internal organ in the body performs a wide range of physiologically important metabolic functions including synthesis of plasma proteins, glycogen storage, fatty acid biosynthesis, bile secretion, detoxification (xenobiotic metabolism) and blood glucose generation etc. It also involves in metabolic homeostasis by regulating carbohydrate, lipid, and amino acids levels and the major site of gluconeogenesis. Unlike in other animals, in Poultry, liver is the major site of de novo fatty acid biosynthesis . However, it is difficult to study specific liver activities in vivo owing to interfering influences from other organs like kidney, gut, and lungs, which metabolize drugs and the muscle involvement in glucose homeostasis. Hence, culturing the liver parenchymal cells in vitro is helpful for us to study the various activities of hepatocytes.

Materials for cell culture

Standard cell culture equipment Laminar flow hood, humidified chamber (CO2 (5%) incubator), Standard inverted microscope, Centrifuge, Water bath.

Liver is one of the important organs in the body that regulates various metabolisms in the body and having multiple functions. It is difficult to study all the functions of the liver in vivo, because of other organs interventions. Hence, the liver parenchymal cells (hepatocytes) were cultured in vitro in order to study the different activities of the liver. In the present study, we standardized culturing of chicken embryo hepatocytes from 12 to 14 days old embryos.

Here we have used 0.125% trypsin EDTA to disaggregate the parenchymal cells of the liver. The cells were cultured using growth media added with 10% Fetal Bovine Serum and Tryptone Phosphate Broth.

Materials required

Pipettes, Gloves, Scissors and forceps, 0.22µm filters, Muslin cloth, Magnetic stirrer, Beakers, 25-cm2 flask, micro pipette tips. Tissue required 12-14 days old chicken embryo Media and the reagents required Phosphate Buffer Saline (PBS), Trypsin EDTA (Gibco by Life Technologies, USA), Growth medium – M-199 medium (Sigma Aldrich, USA) and Fetal Bovine Serum (Gibco by Life Technologies, USA).

Protocol

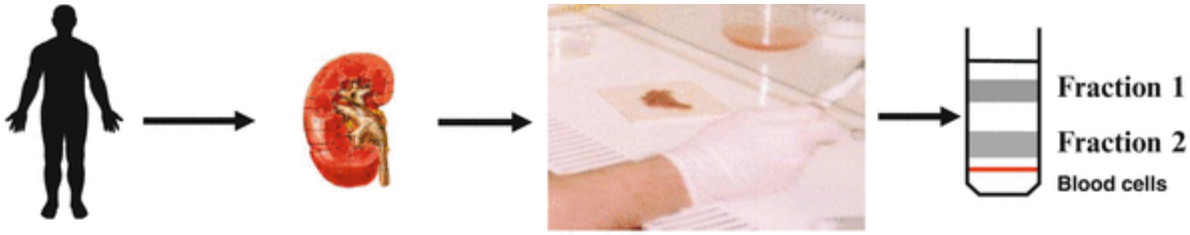
12 to 14 days old embryonated eggs were utilized and at this stage we will get a visible and appreciable amount of liver samples for the culture of hepatocytes. The whole egg was disinfected by swabbing with 70% alcohol and placed in a small beaker with their blunt end facing up. The egg shell was cracked at broad end by using sterile forceps, and white shell membrane was peeled off to expose the chorioallantoic membrane (CAM) with its blood vessels. Further, membranes were pierced with sterile curved forceps and the embryo was raised out by grasping it gently and placed in a 9-cm Petri dish containing autoclaved sterile Phosphate Buffer Saline (PBS) and cleaned thoroughly. The head, limbs and wings of the embryo were detached and the ventral side of the embryo was cut opened by means of sterile scissors. Further, the abdomen portion was separated to locate the liver and the liver lobes were collected aseptically using sterile forceps into another petri dish contained sterile PBS. The liver lobes were separated and the part of hepatic vessels and unnecessary tissue were detached. The gall bladder was removed carefully without piercing. The liver lobes were minced into pieces using sterile scissors and the unwanted tissue, such as membranes, fat or other attached tissues were dissected. After that, the liver pieces were transferred to another petri dish having PBS to clean several times to wash off all the all debris and blood tinge.

Finally, the tissue pieces were cleaned once again by resuspending in a beaker containing PBS and allowed the pieces to settle down and the supernatant was discarded (the same was repeated two more times). These tissue pieces were shifted to a sterile beaker containing sterile magnetic bar and approximately 10-15 mL (maintained at room temperature) of 0.125% trypsin-EDTA (Gibco by Life Technologies, USA) was added for disaggregation of cells. Further, the beaker was placed on magnetic stirrer in an incubator at room temperature for stirring at around 100 RPM for less than 10 minutes. The beaker was taken out from the stirrer and allowed the left over pieces to settle at the bottom. The supernatant was filtered through sterile double layered muslin cloth into a fresh, sterile beaker. The filtrate was taken into a 50 mL tube and centrifuged at

3000 RPM for 5 minutes. The resultant pellet was re-suspended in 5 mL growth medium, M-199 (Sigma-Aldrich, USA) with Fetal Bovine Serum (FBS; Gibco by Life Technologies, USA) to stop the action of trypsin. The cells with medium were centrifuged at 3000 RPM for 3 minutes. The ensuing pellet was re-suspended in 5 mL of growth medium containing 10% FBS, 1% Tryptone Phosphate Broth (Sigma-Aldrich, USA) and Antibiotic Antimycotic solution (100000 units Penicillin, 10mg Streptomycin and 25µg Amphotericin B; HiMedia). The cell suspension was to diluted 1x106 cells /mL in growth medium with the help of hemocytometer or electronic cell counter, and seeded approximately 2x105 cells /cm2 in each 25 cm2 tissue culture flask. The culture flasks were incubated in humidified incubator at 370C temperature with 5% CO2 and 95% air.

Further, the medium was changed at regular intervals (36-48 hours) as dictated by the depression of pH by changing the colour of medium to pink to yellowish. The optimal conditions for hepatocyte proliferation require a balance of necessary components for cellular proliferation. Further, these cells grow in patches and extend from all the sides. Though the cells with the above said protocol can proliferate and survive for 8 to 10 days from the day of culture, further studies are required to maintain the cellular growth and multiplication. Thus, it is critical to optimize cell culture for each experiment type and to use as long as possible identical batch reagents and media.

Primary Culture -Kidney cells (Human)

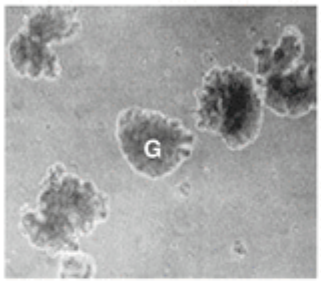


Kidney is removed (usually due to renal carcinoma). A piece of macroscopically healthy region is retained for cell preparation.

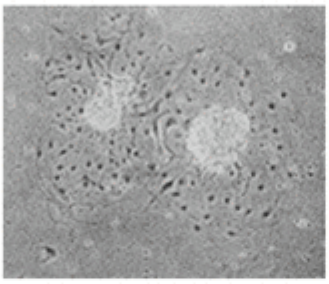
Cortex is isolated and minced into small pieces. Minced material is then incubated in a collagenase solution.

Percoll density centrifugation is carried out.

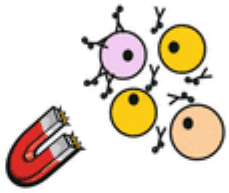
Fraction 1



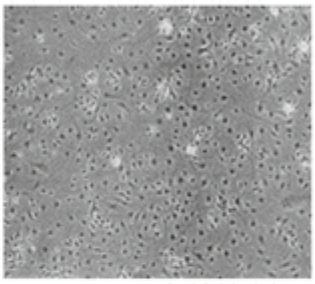
Material removed from fraction 1 consists mostly of glomeruli (G). Glomeruli are subjected to a further collagenase digestion. Digested glomeruli are plated on fibronectin coated dishes.



After 7 days of culture microvascular endothelial cells grow out from the attached glomeruli. Glomerular epithelial cells and mesangial cells are also present.

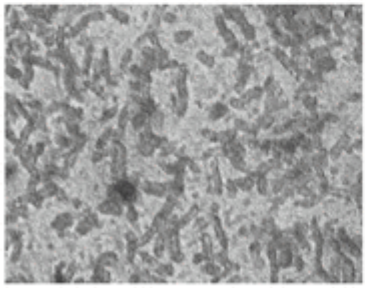


The mixed cell culture is incubated with antibodies against PECAM-1 bound to magnetic beads. PECAM positive cells are isolated with magnetic sorting.



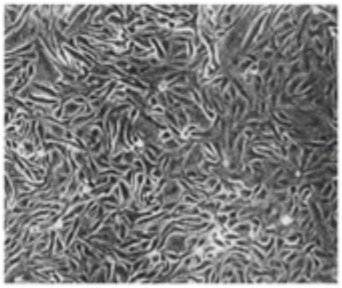
Glomerular microvascular endothelial cells grow to confluence and form a cobblestone morphology. Cells can be passage up to 8 times. (Cells in picture are passage 3).

Fraction 2



Material removed from fraction 2 consists mostly of large proximal tubular fragments.

Fragments are plated onto fibronectin coated dishes. In order to select for proximal tubular cells initial growth medium contains no additional glucose to that contained in fetal calf serum. Also L-valine is replaced with D-valine to prevent fibroblast growth.



Proximal tubular cells grow to confluence within 5 days exhibiting a typical cobblestone morphology. They can be passaged 3 times. (Cells in picture are at passage 0).

Maintenance and Storage of culture

SUBCULTURE AND PROPAGATION

The first *subculture* represents an important transition for a culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate. Hence, cell proliferation has become an important feature. Although the primary culture may have a variable growth fraction,, depending on the type of cells present in the culture, after the first subculture, the growth fraction is usually high (80% or more). From a very heterogeneous primary culture, containing many of the cell types present in the original tissue, a more homogeneous cell line emerges. In addition to its biological significance, this process has considerable practical importance, as the culture can now be propagated, characterized, and stored, and the potential increase in cell number and the uniformity of the cells open up a much wider range of experimental possibilities.

Once a primary culture is subcultured (or *passaged*), it becomes known as a *cell line*. This term implies the presence of several cell lineages of either similar or distinct phenotypes. If one cell lineage is selected, by cloning , by physical cell, or by any other selection technique, to have certain specific properties that have been identified in the bulk of the cells in the culture, this cell line becomes known as a *cell strain.*

If a cell line transforms *in vitro*, it gives rise to a *continuous cell line* , and if selected or cloned and characterized, it is known as a *continuous cell strain*. The first subculture gives rise to a *secondary* culture, the secondary to a *tertiary*, and so on, although in practice, this nomenclature is seldom used beyond the tertiary culture. Each subculture divided the culture in half (i.e., the *split ratio* was 1:2), so passage number was the same as generation number. However, they need not be the same. The *passage number* is the number of times that the culture has been subcultured, whereas the *generation number* is the number of doublings that the cell population has undergone, given that the number of doublings in the primary culture is very approximate. When the split ratio is 1:2, the passage number is approximately equal to the generation number.

TABLE 13.1. Commonly Used Cell Lines

Cell line	Morphology	Origin	Species	Age	Ploidy	Characteristics	Reference
Finite, from Normal Tissue							
IMR-90	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Nichols et al., 1977
MRC-5	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Jacobs, 1970
MRC-9	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Jacobs, et al., 1979
WI-38	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection	Hayflick & Moorhead, 1961
Continuous, from Normal Tissue							
293	Epithelial	Kidney	Human	Embryonic	Aneuploid	Readily transfected.	Graham et al., 1977
3T3-A31	Fibroblast		Mouse BALB/c	Embryonic	Aneuploid	Contact inhibited; readily transformed	Aaronson & Todaro 1968
3T3-L1	Fibroblast		Mouse Swiss	Embryonic	Aneuploid	Adipose differentiation	Green & Kehinde, 1974
BEAS-2B	Epithelial	Lung	Human	Adult			Reddel et al., 1988
BHK21-C13	Fibroblast	Kidney	Syrian hamster	Newborn	Aneuploid	Transformable by polyoma	Macpherson & Stoker, 1962
BRL 3A	Epithelial	Liver	Rat	Newborn		Produce IGF-2	Coon, 1968
C2	Fibroblastoid	Skeletal muscle	Mouse	Embryonic		Myotubes	Morgan et al., 1992
C7	Epithelioid	Hypothalamus	Mouse			Neurophysin; vasopressin	De Vitry et al., 1984
CHO-K1	Fibroblast	Ovary	Chinese hamster	Adult	Diploid	Simple karyotype	Puck et al., 1958
COS-1, COS-7	Epithelioid	Kidney	Pig	Adult		Good hosts for DNA transfection	Gluzman, 1981
CPAE	Endothelial	Pulmonary-artery endothelium	Cow	Adult	Diploid	Factor VIII, Angiotensin II converting enzyme	Del Vecchio & Smith, 1981
HaCaT	Epithelial	Keratinocytes	Human	Adult	Diploid	Cornification	Boukamp et al., 1988
L6	Fibroblastoid	Skeletal muscle	Rat	Embryonic		Myotubes	Richler & Yaffe, 1970
LLC-PK1	Epithelial	Kidney	Pig	Adult	Diploid	Na^+-dependent glucose uptake	Hull et al., 1976; Saier, 1984
MDCK	Epithelial	Kidney	Dog	Adult	Diploid	Domes, transport	Gaush et al., 1966; Rindler et al., 1979
NRK49F	Fibroblast	Kidney	Rat	Adult	Aneuploid	Induction of suspension growth by TGF-α,β	De Larco & Todaro, 1978
STO	Fibroblast		Mouse	Embryonic	Aneuploid	Used as feeder layer for embryonal stem cells	Bernstein, 1975
Vero	Fibroblast	Kidney	Monkey	Adult	Aneuploid	Viral substrate and assay	Hopps et al., 1963
Continuous, from Neoplastic Tissue							
A2780	Epithelial	Ovary	Human	Adult	Aneuploid	Chemosensitive with resistant variants	Tsuruo et al., 1986
A549	Epithelial	Lung	Human	Adult	Aneuploid	Synthesizes surfactant	Giard et al., 1972
A9	Fibroblast	Subcutaneous	Mouse	Adult	Aneuploid	Derived from L929; Lacks HGPRT.	Littlefield, 1964b
B16	Fibroblastoid	Melanoma	Mouse	Adult	Aneuploid	Melanin	Nilos & Makarski, 1978
C1300	Neuronal	Neuroblastoma	Rat	Adult	Aneuploid	Neurites	Liebermann & Sachs, 1978
C6	Fibroblastoid	Glioma	Rat	Newborn	Aneuploid	Glial fibrillary acidic protein, GPDH	Benda et al., 1968

CULTURE AGE

Cell lines with limited culture life spans are known as *finite* cell lines and behave in a fairly reproducible fashion . They grow through a limited number of cell generations, usually between 20 and 80 cell population doublings, before extinction. The actual number of doublings depends on species and cell lineage differences, clonal variation, and culture conditions, but it is consistent for one cell line grown under the same conditions. It is therefore important that reference to a cell line should express the approximate generation number or number of doublings since explantation;

Continuous cell lines have escaped from senescence control, so the generation number becomes less important and the number of passages since last thawed from storage becomes more important .In addition, because of the increased cell proliferation rate and saturation density ,split ratios become much greater (1:20–1:100) and cell concentration at subculture becomes much more critical .

CELL LINE DESIGNATIONS

New cell lines should be given a code or designation [e.g., normal human brain (NHB)]; a cell strain or cell line number (if several cell lines were derived from the same source; e.g., NHB1, NHB2, etc.); and, if cloned, a clone number (e.g., NHB2-1, NHB2-2, etc.). It is useful to keep a log book or computer database file where the receipt of biopsies or specimens is recorded before initiation of a culture. The accession number in the log book or database file, perhaps linked to an identifier letter code, can then be used to establish the cell line designation; for example, LT156 would be lung tumor biopsy number 156. This method is less likely to generate ambiguities, such as the same letter code being used for two different cell lines, and gives automatic reference to the record of accession of the line. Rules of confidentiality preclude the use of a donor's initials in naming a cell line.

For finite cell lines, the number of population doublings should be estimated and indicated after a forward slash, e.g., NHB2/2, and increases by one for a split ratio of 1:2 (e.g., NHB2/2, NHB2/3, etc.), by two for a split ratio of 1:4 (e.g., NHB2/2, NHB2/4, etc.), and so on. When dealing with a continuous cell line a ''p'' number at the end is often used to indicate the number the number of passages since the last thaw from the freezer , e.g., HeLa-S3/p4.

CHOOSING A CELL LINE :

The general parameters to consider in selecting a cell line are :

(1) **Finite vs. Continuous.** Is there a continuous cell line that expresses the right functions?

A continuous cell line generally is easier to maintain, grows faster, clones more easily, produces a higher cell yield per flask, and is more readily adapted to serum-free medium (*see* Table 13.3).

(2) **Normal or Transformed.** Is it important whether the line is malignantly transformed or not? If it is, then it might be possible to obtain an immortal line that is not tumorigenic, e.g., 3T3 cells or BKK21-C13.

(3) **Species.** Is species important? Nonhuman cell lines have fewer biohazard restrictions and have the advantage that the original tissue may be more accessible.

(4) **Growth Characteristics.** What do you require in

terms of growth rate, yield, plating efficiency, and ease of harvesting? You will need to consider the following parameters:

a) Population-doubling time

b) Saturation density

c) Plating efficiency

d) Growth fraction

e) Ability to grow in suspension

(5) **Availability.** If you have to use a finite cell line, are there sufficient stocks available, or will you have to generate your own line(s)? If you choose a continuous cell line, are authenticated stocks available?

(6) **Validation.** How well characterized is the line (*see* Section 7.10), if it exists already, or, if not, can you do the necessary characterization (*see* Chapter 16)? Is the line authentic (*see* Section 16.3)? It is vital to eliminate the possibility of cross-contamination before embarking on a program of work with a cell line, as so many cross-contaminations have

been reported (*see* Table 13.2).

(7) **Phenotypic Expression.** Can the line be made to express the right characteristics (*see* Section 17.7)?

(8) **Control Cell Line.** If you are using a mutant, transfected, transformed, or abnormal cell line, is there a normal equivalent available, should it be required?

(9) **Stability.** How stable is the cell line .

Significance of Cell Morphology :

Whatever procedure is undertaken, it is vital that the culture be examined carefully to confirm the absence of contamination . The cells should also be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate . Such signs may imply that the culture requires a medium change, or may indicate a more serious problem, e.g., inadequate or toxic medium or serum, microbial contamination, or senescence of the cell line. Medium deficiencies can also initiate apoptosis.

Replacement of Medium

Four factors indicate the need for the replacement of culture medium:

A Drop in pH. The rate of fall and absolute level should be considered. Most cells stop growing as the pH falls from pH 7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed. Try to estimate the rate of fall; a culture at pH 7.0 that falls 0.1 pH units in one day will not come to harm if left a day or two longer before feeding, but a culture that falls 0.4 pH units in one day will need to be fed within 24–48 h and cannot be left over a weekend without feeding.

Cell Concentration. Cultures at a high cell con-centration exhaust the medium faster than those at a low concentration. This factor is usually evident in the rate of change of pH, but not always.

Cell Type. Normal cells (e.g., diploid fibroblasts) usually stop dividing at a high cell density , because of cell crowding, growth factor depletion, and other reasons. The cells block in the G_1

phase of the cell cycle and deteriorate very little, even if left for two to three weeks or longer. Transformed cells, continuous cell lines, and some embryonic cells, however, deteriorate rapidly at high cell densities unless the medium is changed daily or they are subcultured.

Morphological Deterioration. This factor must be anticipated by regular examination and familiarity with the cell line .If deterioration is allowed to progress too far, it will be irreversible, as the cells will tend to enter apoptosis .

CRITERIA FOR SUBCULTURE :

The need to subculture a monolayer is determined by the following criteria:

(1) **Density of Culture.** Normal cells should be subcultured as soon as they reach confluence. If left more than 24 h, they will withdraw from the cycle and take longer to recover when reseeded. Transformed cells should also be subcultured on reaching confluence; although they will continue to proliferate beyond confluence, they will start to deteriorate after about two doublings, and reseeding efficiency will decline.

(2) **Exhaustion of Medium.** Exhaustion of the medium usually indicates that the medium requires replacement, but if a fall in pH occurs so rapidly that the medium must be changed more frequently, then subculture may be required. Usually, a drop in pH is accompanied by an increase in cell density, which is the prime indicator of the need to subculture. Note that a sudden drop in pH can also result from contamination, so be sure to check .

(3) **Time Since Last Subculture.** Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved and monitored. If cells have not reached a high-enough density (i.e., they are not confluent) by the appropriate time, then increase the seeding density, or if they reach confluence too soon, then reduce the seeding density. Once this routine is established, the recurrent growth should be consistent in duration and cell yield from a given seeding density. Deviations from this pattern then signify a departure from normal conditions or indicate deterioration of the cells. Ideally, a cell concentration should be found that allows for the cells to be subcultured after 7 days, with the medium being changed after 3–4 days.

Requirements for Other Procedures. When cells are required for purposes other than routine propagation, they also have to be subcultured, in order to increase the stock or to change the type of culture vessel or medium. Ideally, this procedure should be done at the regular subculture time, when it will be known that the culture is performing routinely, what the reseeding conditions should be, and what outcome can be expected. However, demands for cells do not always fit the established routine for maintenance, and compromises have to be made, but (1) cells should not be subcultured while still within the lag period, and (2) cells should always be taken between the middle of the log phase and the time before which they have entered the plateau phase of a previous subculture (unless there is a specific requirement for plateau-phase cells, in which case they will need frequent feeding or continuous perfusion).

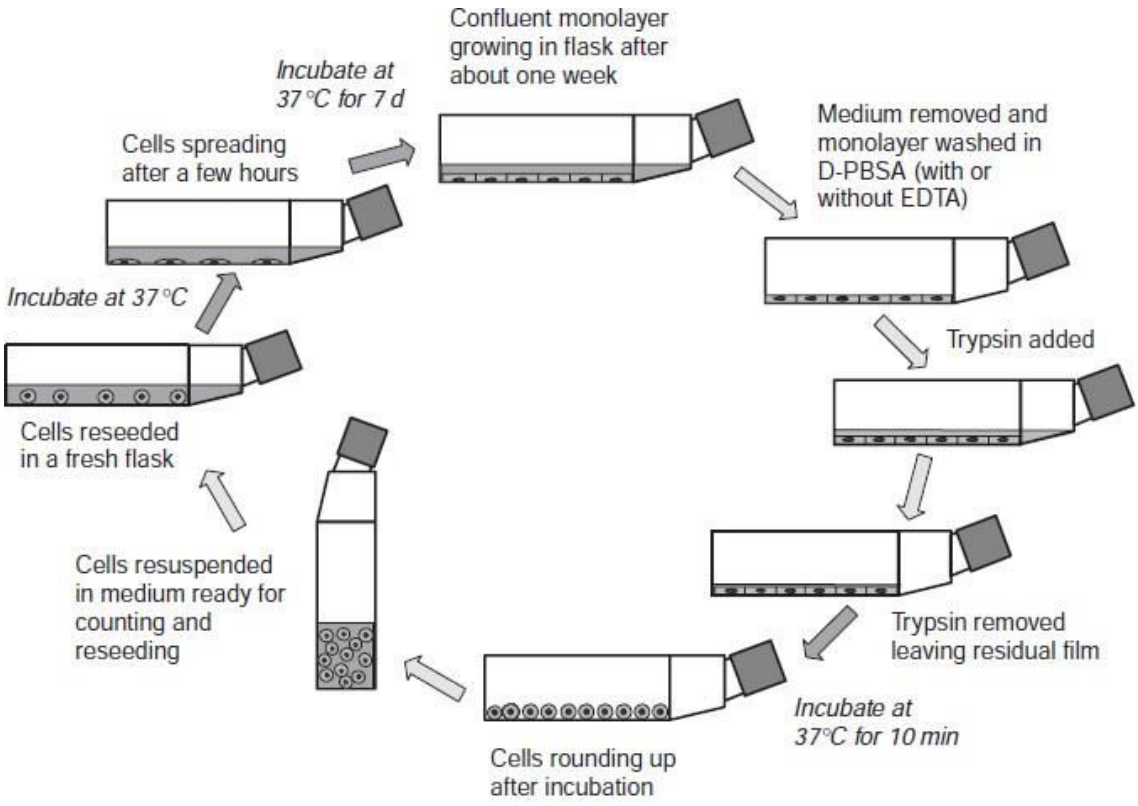


Fig. 13.3. Subculture of Monolayer. Stages in the subculture and growth cycle of monolayer cells after trypsinization (*see also* Plates 4, 5).

PROPAGATION IN SUSPENSION :

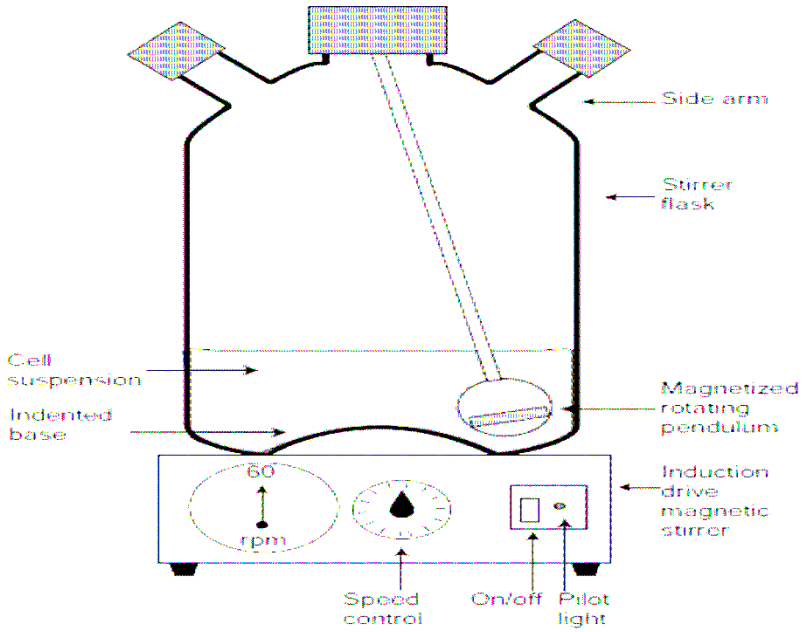


Fig. 13.5. Stirrer Culture. A small stirrer flask, based on the Techne design, with a capacity of 250—1000 mL. The cell suspension is stirred by a pendulum, which rotates in an annular depression in the base of the flask.

As a general rule, most continuous cell lines subculture satisfactorily at a seeding concentration of between 1×10^4 and 5×10^4 cells/mL.

The criteria for subculture are similar to those for monolayers:

(1) **Cell Concentration,** which should not exceed 1×10^6 cells/mL for most suspension-growing cells.

(2) **pH,** which is linked to cell concentration, and declines as the cell concentration rises.

(3) **Time Since Last Subculture,** which, as for monolayers, should fit a regular schedule.

(4) **Cell Production Requirements** for experimental or production purposes.

CELL CLONING :

The traditional microbiological approach to the problem of culture heterogeneity is to isolate pure cell strains by cloning, but, although this technique is relatively easy for continuous cell lines, its success in most primary cultures is limited by poor cloning efficiencies. Cloning of attached cells may be carried out in Petri dishes, multiwell plates, or flasks, and it is relatively easy to discern individual colonies. Micromanipulation is the only conclusive method for

determining genuine clonality (i.e., that a colony was derived from one cell), but when symmetrical colonies are derived from a single-cell suspension, particularly if colony formation is monitored at the early stages, then it is probable that the colonies are clones.

Cloning can also be carried out in suspension by seeding cells into a gel, such as agar or agarose, or a viscous solution, such as Methocel, with an agar or agarose underlay. The stability of the gel, or viscosity of the Methocel, ensures that daughter cells do not break away from the colony as it forms. Even in monolayer cloning, some cell lines, such as HeLa-S_3 and CHO, are poorly attached, and cells can detach from colonies as they form and generate daughter colonies, which will give an erroneous plating efficiency. This can be minimized by cloning in Methocel without an underlay and allowing the cells to sediment on to the plastic growth surface. Hematopoietic cells are usually cloned in suspension; depending on the cells and growth factors used, the colony generates undifferentiated cells with high repopulation efficiency, *in vivo* or *in vitro*, or may mature into colonies of differentiated hematopoietic cells with very littlerepopulation efficiency. Cloning then becomes an assay for reproductive potential and stem cell identity.

Continuous cell lines generally have a high plating efficiency in monolayer and in suspension because of their transformed status, whereas normal cells, which may have a moderately high cloning efficiency in monolayer, have a very low cloning efficiency in suspension, because of their need to attach and spread out to enter the cell proliferation cycle. Dilution cloning [Puck & Marcus, 1955] is the technique that is used most widely, based on the observation that cells diluted below a certain density form discrete colonies.

STIMULATION OF PLATING EFFICIENCY :

When cells are plated at low densities, the rate of survival falls in all but a few cell lines. This does not usually present a severe problem with continuous cell lines, for which the plating efficiency seldom drops below 10%, but with primary cultures and finite cell lines, the plating efficiency may be quite low—0.5–5%, or even zero. Numerous attempts have been made to improve plating efficiencies, based on the assumption either that cells require a greater range of nutrients at low den-sities, because of loss by leakage, or that cell-derived diffusible signals or conditioning factors are present in high-density cul-tures and are absent or too dilute at low

densities. The intra-cellular metabolic pool of a leaky cell in a dense population will soon reach equilibrium with the surrounding medium, but that of an isolated cell never will. This principle was the basis of the capillary technique of Sanford et al. [1948], by which the L929 clone of L-cells was first produced. The con-fines of the capillary tube allowed the cell to create a locally enriched environment that mimicked a higher cell concentra-tion. In microdrop techniques developed later, the cells were seeded as a microdrop under liquid paraffin, again maintaining a relatively high cell concentration, keeping one colony sep-arate from another, and facilitating subsequent isolation. As media improved, however, plating efficiencies increased, and Puck and Marcus [1955] were able to show that cloning cells by simple dilution (as described in Protocol 14.1) in associa-tion with a feeder layer of irradiated mouse embryo fibroblasts (*see* Protocol 14.3) gave acceptable cloning efficiencies, although subsequent isolation required trypsinization from within a collar placed over each colony (*see* Protocol 14.6).

Conditions that Improve Clonal Growth

(1) **Medium.** Choose a rich medium, such as Ham's F12, or a medium that has been optimized for the cell type in use (e.g., MCDB 110 [Ham, 1984] for human fibroblasts, Ham's F12 or MCDB 302 for CHO [Ham, 1963; Hamilton & Ham, 1977]) (*see* Sections 9.6, 10.5, Tables 10.1, 10.2 and Chapter 23).

(2) **Serum.** When serum is required, fetal bovine is generally better than calf or horse.

(3) Select a batch for cloning experiments that gives a high plating efficiency during tests (*see* Protocol 21.10).

(4) **Hormones.** Insulin, 1×10^{-10} IU/mL, has been found to increase the plating efficiency of several cell types [Hamilton & Ham, 1977]. Dexamethasone, 2.5×10^{-5} M, 10 µg/mL, a soluble synthetic hydrocortisone analog, improves the plating efficiency.

(5) **Carbon Dioxide.** CO_2 is essential for obtaining maximum cloning efficiency for most cells. Although 5% CO_2 is usually used, 2% is sufficient for many cells and may even be slightly better for human glia and fibroblasts. HEPES (20 mM) may be used with 2% CO_2, protecting the cells against pH fluctuations during feeding and in the event of failure of the CO_2 supply. Using 2% CO_2 also cuts down on the consumption of CO_2. At the other extreme, Dulbecco's modification of Eagle's Basal

(6) Medium (DMEM) is normally equilibrated with 10% CO_2 and is frequently used for cloning myeloma hybrids for monoclonal antibody production. The concentration of bicarbonate must be adjusted if the CO_2 tension is altered, so that equilibrium is reached

at pH 7.4 (*see* Table 9.1).

(7) **Treatment of Substrate.** Polylysine improves the plating efficiency of human fibroblasts in low serum concentrations

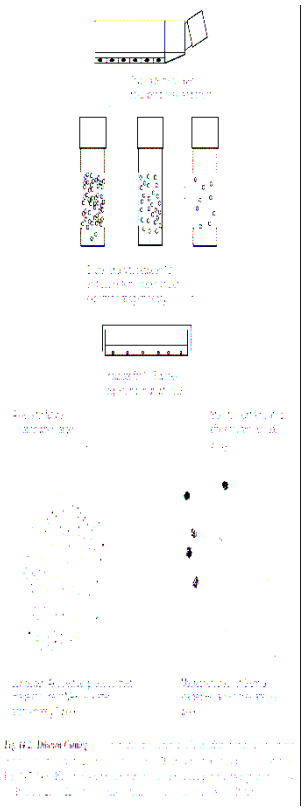
(8) Trypsin.

Conditioned Medium ;

Medium that has been used for the growth of other cells acquires metabolites, growth factors, and matrix products from these cells. This conditioned medium can improve the plating efficiency of some cells if it is diluted into the regular growth medium.

Feeder Layers :

The reason that some cells do not clone well is related to their inability to survive at low cell densities. One way to maintain cells at clonogenic densities but, at the same time, to mimic high cell densities, is to clone the cells onto a growth-arrested feeder layer . The feeder cells may provide nutrients, growth factors, and matrix constituents that enable the cloned cells to survive more readily.



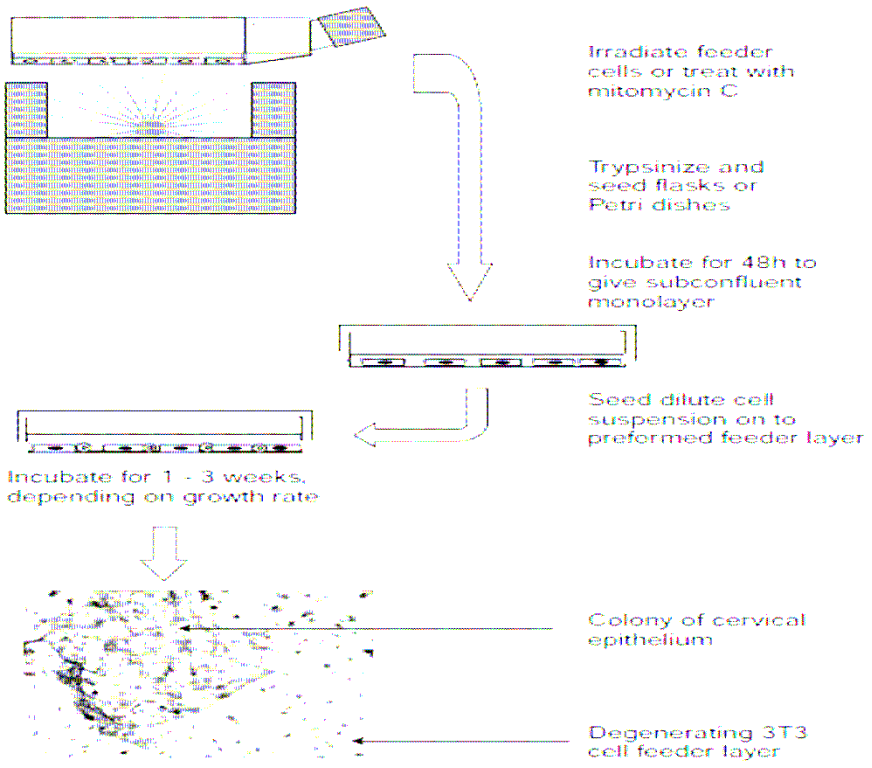


Fig. 14.4. Feeder Layers. Cells are irradiated and trypsinized (or may be trypsinized first and then irradiated in suspension, or treated with mitomycin C) and seeded at a low density to enhance cloning efficiency. (Photo courtesy of M. G. Freshney.)

SUSPENSION CLONING

Some cells, particularly hematopoietic stem cells and virally transformed fibroblasts, clone readily in suspension. To hold the colony together and prevent mixing, the cells are suspended in agar or Methocel and plated on an agar underlay or into dishes that need not be treated for tissue culture.

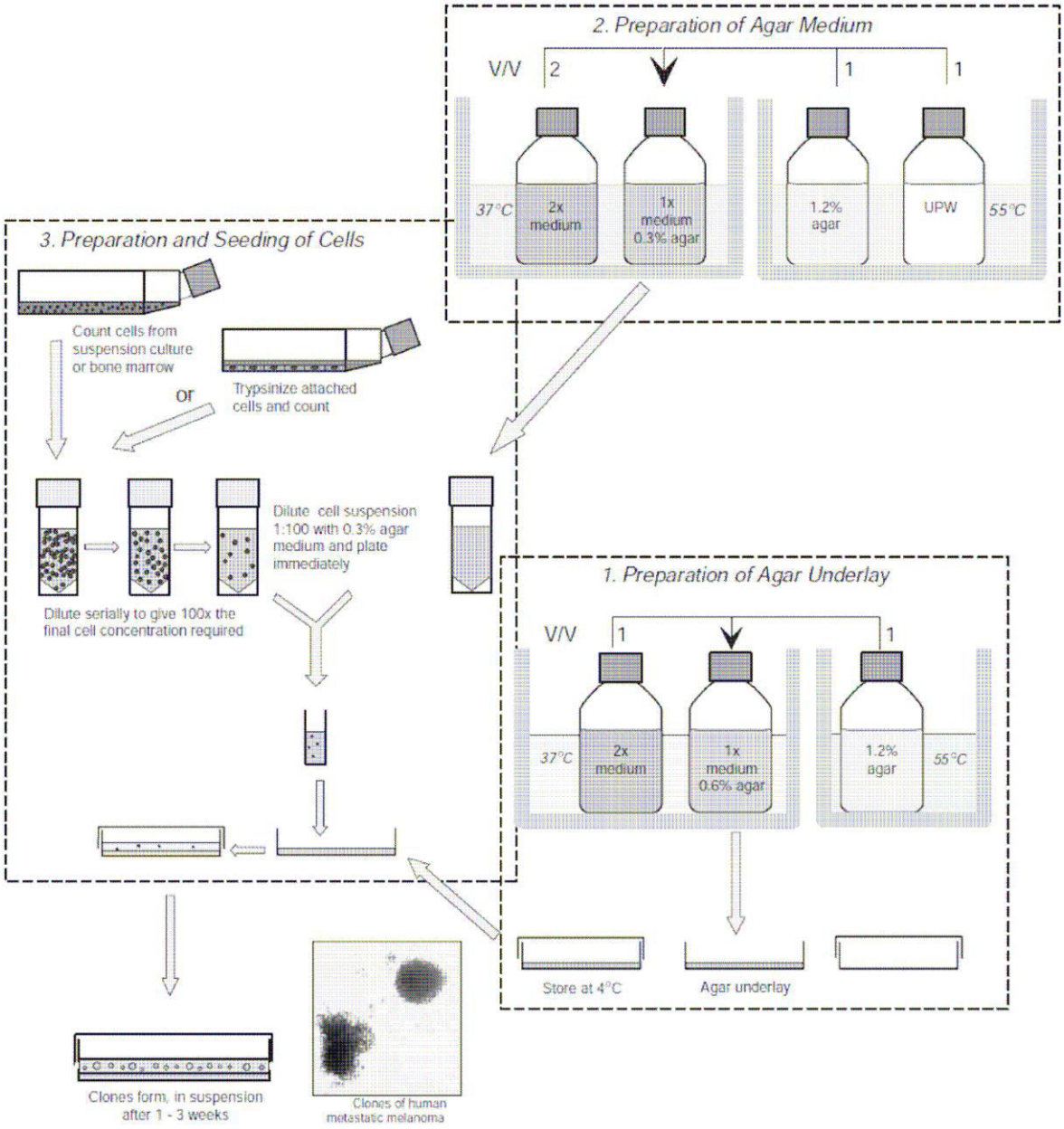


Fig. 14.5. Cloning in Suspension. Cultured cells or primary suspensions from bone marrow or tumors, suspended in agar or low-melting-temperature agarose, which is then allowed to gel, form colonies in suspension. Use of an underlay prevents attachment to the base of the dish. 1. Preparation of agar underlay: Agar, 1.2%, at 55°C is mixed with 2× medium at 37°C and dispensed immediately into dishes, where it is allowed to gel at room temperature or 4°C. 2. Preparation of agar medium: Agar, 1.2%, and UPW are maintained at 55°C and mixed with 2× medium to give 0.3% agar for cloning. The use of low-melting-point agarose allows all solutions to be maintained at 37°C, but this agarose can be more difficult to gel. 3. Cells grown in suspension, derived from bone marrow, or trypsinized from an attached monolayer are counted and diluted serially, and the final product is diluted with agar or agarose and seeded onto an agar underlay.

ISOLATION OF CLONES :

If monolayer cells are cloned directly into multiwell plates (,then colonies may be isolated by trypsinizing individual wells. If cloning is performed in Petri dishes, there is no physical separation between colonies. This separation must be created by removing the medium and placing a stainless steel or ceramic ring around the colony to be isolated.

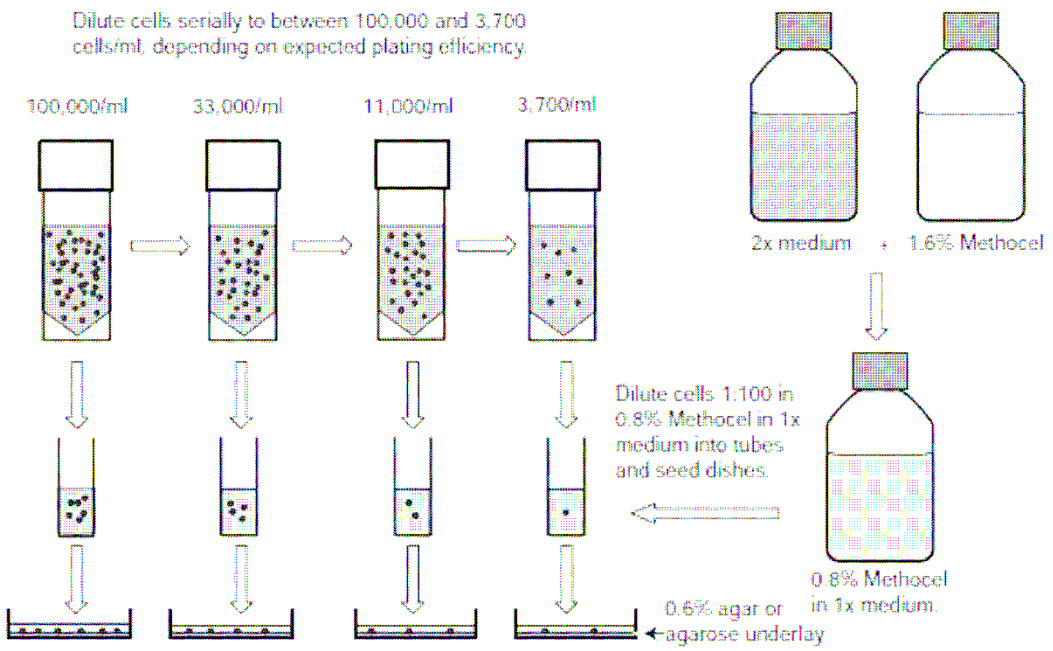


Fig. 14.6. Cloning in Methocel. A series of cell dilutions is prepared as for agar cloning, diluted 1:100 in Methocel medium, and plated into non-tissue-culture-grade dishes or dishes with an agar underlay.

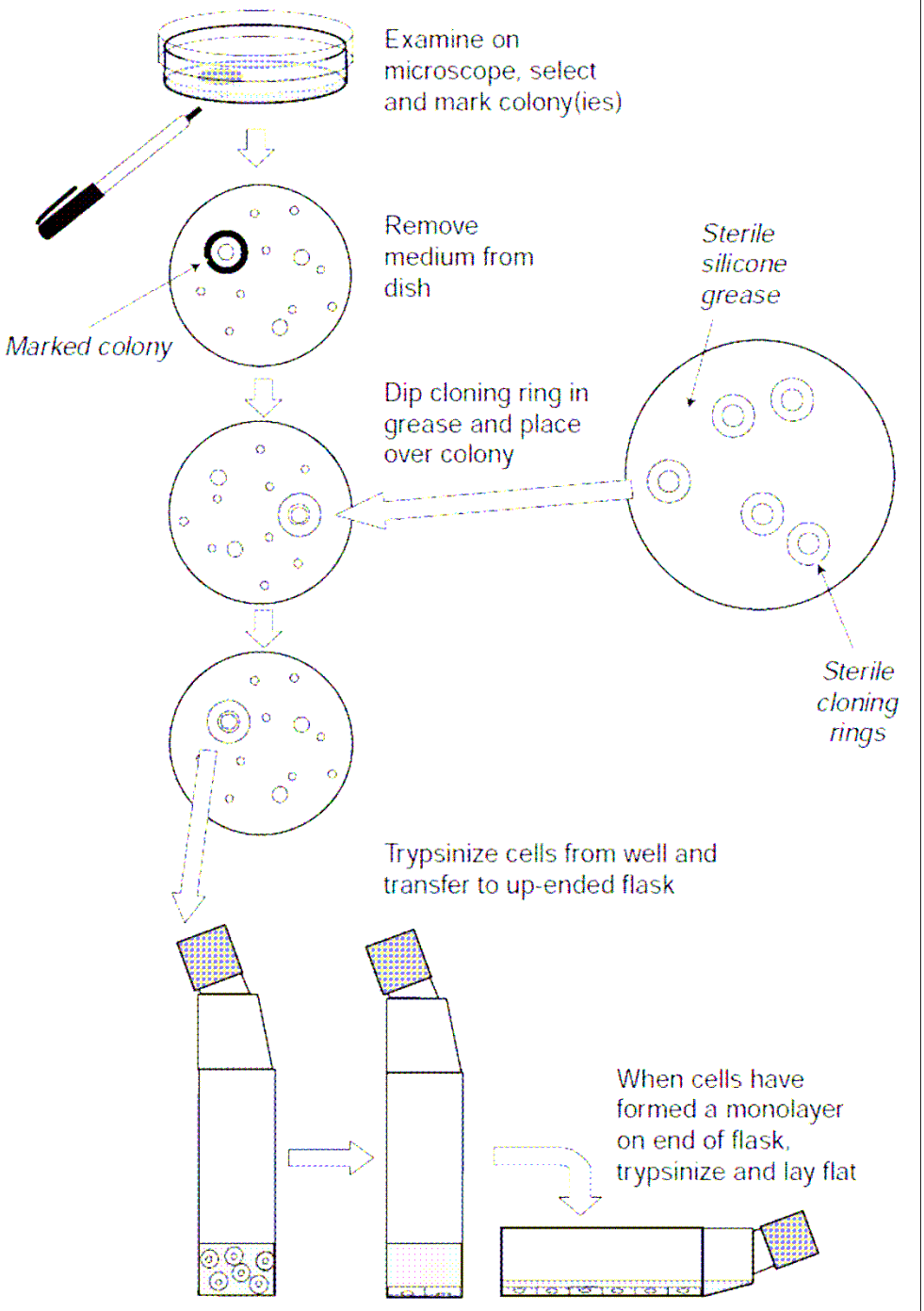


Fig. 14.8. Isolation of Monolayer Clones. The mature colonies are examined on the microscope.

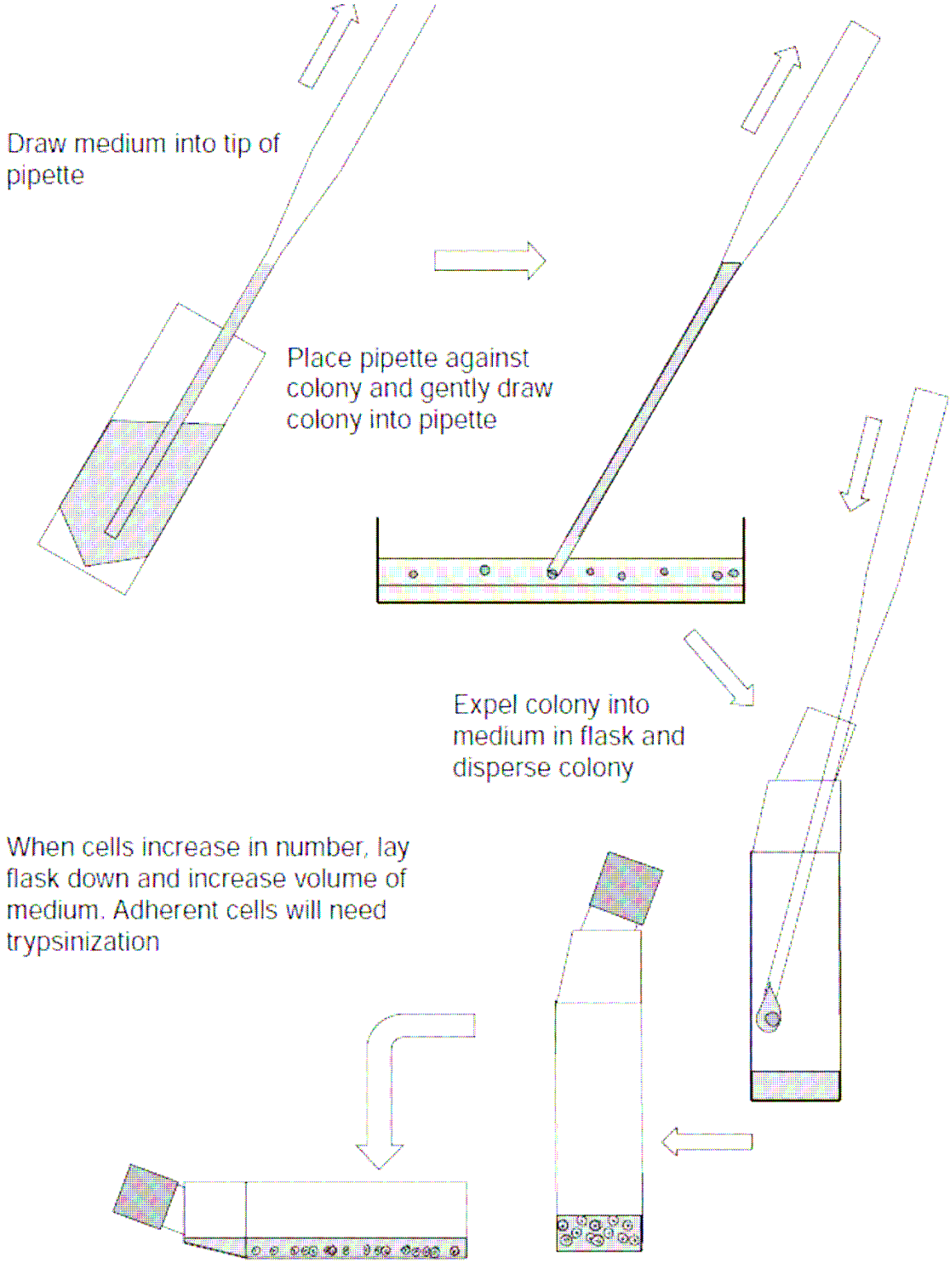
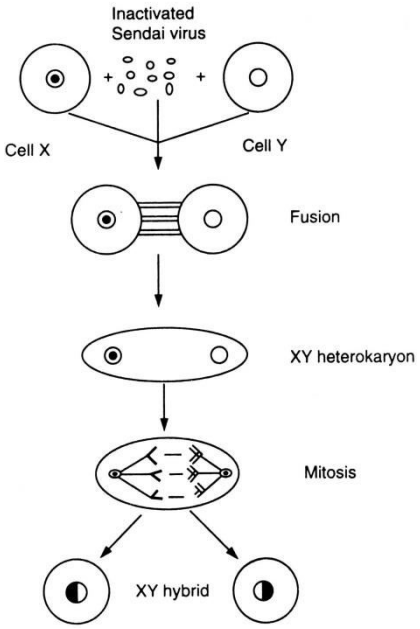


Fig. 14.9. Isolation of Suspension Clones. Mark the colony as for monolayer, then draw the colony into a Pasteur pipette (or pipettor tip). Transfer the colony to a culture flask, disperse it in medium, and incubate it. Make up medium when cells start to grow.

Somatic Cell Fusion

In animals fusion of two different cells and production of a hybrid cell have been successfully achieved. These hybrid cells have significant biotechnological applications in many more areas such as: *(i)* study of control of gene expression and differentiation, *(ii)* gene mapping, *(iv)* malignancy, *(iv)* viral replication, and *(v)* antibody production through hybridoma technology. In 1960s, in France, the hybrid cells were successfully produced from mixed cultures of two different cell lines of mouse.

Moreover, within the body fusion of myoblasts and formation of multinucleate fibers may be exemplified. They can also be allowed to fuse *in vitro* and form heterokaryons. Macrophages fuse around the foreign body or bacterial cells in the tissues. Bone cells are also known to undergo somatic cell fusion. Cells growing in culture are induced by some of the viruses such as 'Sendai virus' to fuse and form hybrids. This virus induces two different cells first to form heterokaryon (Fig. 6.7). During mitosis chromosome of heterokaryons are brought towards two poles which later on fuse to form hybrids. Removal of surface carbohydrates is necessary before establishment of cell fusion. Some chemicals such as polyethylene glycol also induce somatic cell fusion. It is interesting to note that the cells of taxonomically different animals can fuse and form hybrids. This suggests that there is no compatibility between membranes, nuclei, organelles of two different groups of animal cells (Sidebottom and Ringertz, 1984).



STEM CELLS CULTURES:

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can divide (through mitosis) to produce more stem cells. They are found in multicellular organisms. In mammals, there are two broad types of stem cells: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues. In a developing embryo, stem cells can differentiate into all the specialized cells—ectoderm, endoderm and mesoderm (see induced pluripotent stem cells)—but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues.

There are three known accessible sources of autologous adult stem cells in humans:

1. Bone marrow, which requires extraction by *harvesting*, that is, drilling into bone (typically the femur or iliac crest).
2. Adipose tissue (lipid cells), which requires extraction by liposuction.
3. Blood, which requires extraction through apheresis, wherein blood is drawn from the donor (similar to a blood donation), and passed through a machine that extracts the stem cells and returns other portions of the blood to the donor.

Stem cells can also be taken from umbilical cord blood just after birth. Of all stem cell types, autologous harvesting involves the least risk. By definition, autologous cells are obtained from one's own body, just as one may bank his or her own blood for elective surgical procedures.

Adult stem cells are frequently used in medical therapies, for example in bone marrow transplantation. Stem cells can now be artificially grown and transformed (differentiated) into specialized cell types with characteristics consistent with cells of various tissues such as muscles or nerves. Embryonic cell lines and autologous embryonic stem cells generated through Somatic- cell nuclear transfer or dedifferentiation have also been proposed as promising candidates for future therapies.

Stem Cells are different from other cells because:

1. They can continue to divide for long periods of time: Most cells such as skin cells cannot replicate themselves after a certain period of time. Stem cells are self-sustaining by replicating themselves for a much longer period of time.

2. They are unspecialized: Specialized cells have specific capabilities that allow them to perform certain tasks. For example a red blood cell contains hemoglobin that allows it to carry oxygen. Stem cells have unspecialized capability and do not have tissue- specific structures to perform specialized functions.

3. They can give rise to specialized cells: Stem cells go through a process called **differentiation** and create special types of cells (muscle, nerve, skin, etc.).

Embryonicstemcells Embryonic stem cells are the cells within the protective layer of the blastocyst. They are pluripotent, which means they can develop into any of the cells of the adult body. Researchers believe that, because they are pluripotent, and easy to grow, they have the best potential for replacing damaged or lost tissue or body parts.

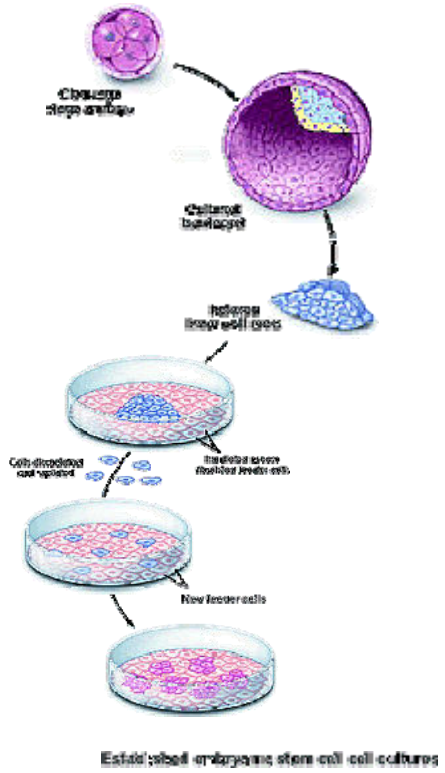
Adultstemcells Also known as **progenitor cells** or **somatic stem cells**, adult stem cells are located, in small quantities, throughout the body and generate specialized cells for the area they are located. These cells do not renew themselves as well as embryonic stem cells. Still, if these cells are put in a different environment, they may produce a different type of cells from the originating cell.

Stem cell research is an active area of inquiry and scientists are discovering new characteristics of stem cells every day. For example, recent research indicated that multipotent stem cells from one type of tissue (blood) might actually have the ability to generate cells for a different type of tissue (nerve).

Scientists are continuing to search for new sources of adult stem cells. Some of the locations where stem cells have been located include: bone marrow, skin, liver, blood, and the brain. Some adult stem cells, which have already been used to treat illnesses, include **hematopoietic** stem cells and umbilical cord blood stem cells.

Hematopoietic stem cells are located in the bone marrow and form blood cells. They have been successfully used to treat blood disorders for younger patients. **Umbilical**

cord blood stem cells are located in the blood of the umbilical cord after birth. Umbilical cord stem cells are similar to hematopeitetic stem cells in adults, but they are less mature and have much more potential to differentiate into various types of cells.



WHOLE EMBRYO CULTURE

Whole embryo culture appears to be an excellent method to screen chemicals for teratogenic hazard. Compared to *in* vivotesting it is cheap and rapid and does not involve experimentation on live adult animals. Also in the important area of risk estimation whole embryo culture offers distinct advantages over *in vivo* teratogenicity testing. Adverse embryonic outcomes (malformations or embryotoxicity) are directly related to the serum concentration of the compound being tested and can be compared to the serum concentration in the human. A similar comparison is not possible after *in vivo* testing because for most compounds there are major pharmacokinetic differences between humans and experimental animals. *In vivo* testing is also limited by the possibility that metabolites that occur

in the human do not occur in the test animal. This problem can be overcome in the *in vitro* system by adding the metabolite directly atthe desired concentration either with or without the parent compound. There is only one major disadvantage to *in vitro* testing and that is the limited period of embryogenesis that is undertaken in the commonly used culture system. This restricts the range of malformations that can be induced and may render the testing system unsuitable for compounds that are likely to exert their major toxicological effect late in gestation. Any evaluation of whole embryo culture for hazard and risk assessment in teratology must take into account the limited value of currently used *in vivo* methods. Over 2000 chemicals have been reported to be teratogenic in experimental animals exposed *in vivo*. In comparison only about 20 chemicals are known to cause birth defects in the human. This large number of *in vivo* false positive cannot easily be distinguished from true- positives. In this respect *in vivo* testing is severely deficient. The embryo culture testing system would also be expected to produce many falsepositives; but by comparing effective drug concentrations with human therapeutic concentrations they can be differentiated from true- positives. The most serious deficiency for an *in vivo* or *in vitro* teratogenicity testing system would be false-negatives. This has not been a problem in the validation of *in vitro* testing so far (except perhaps procarbazine), but difficult drugs such as thalidomide were not included. Thalidomide remains an important index chemical because it is not teratogenic in rats or mice but is teratogenic in the rabbit and human. It is likely that these species differences are due to metabolic differences between species and it is possible that if the proximate *teratogen/s* of thalidomide were identified they would be teratogenic in rat embryo culture. Whole embryoculture remains a very powerful technique that should continue to contribute to the determination of the safety of drugs and other chemicals during pregnancy.

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SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – ANIMAL BIOTECHNOLOGY – SBT1305

UNIT II GROWTH AND SCALE UP

Cell growth characteristics and kinetics, Micro-carrier attached growth, Cell culture in continuous, perfusion and hollow fibre reactor.

THE NEED FOR CHARACTERIZATION :

There are six main requirements for cell line characterization:

(1) Demonstration of the absence of cross-contamination .

(2) Confirmation of the species of origin

(3) Correlation with the tissue of origin, which comprises the following characteristics:

a) Identification of the lineage to which the cell belongs

b) Position of the cells within that lineage (i.e., the stem, precursor, or differentiated status)

(4) Determination of whether the cell line is transformed or not:

a) Is the cell line finite or continuous

b) Does it express properties associated with malignancy

(5) Indication of whether the cell line is prone to genetic instability and phenotypic variation

(6) Identification of specific cell lines within a group from the same origin, selected cell strains, or hybrid cell lines, all of which require demonstration of features unique to that cell line or cell strain.

Characterization of a cell line is vital, not only in determining its functionality but also in proving its authenticity; special attention must be paid to the possibility that the cell line has become cross-contaminated with an existing continuous cell line or misidentified because of

mislabeling or confusion in handling.

TABLE 16.1. Characterization of Cell Lines and Cell Strains

Criterion	Method
Karyotype	Chromosome spread with banding
Isoenzyme analysis	Agar gel electrophoresis
Cell surface antigens	Immunohistochemistry
Cytoskeleton	Immunocytochemistry with antibodies to specific cytokeratins
DNA fingerprint	Restriction enzyme digest; PAGE; satellite DNA probes
DNA Profile	PCR of microsatellite repeats

SPECIES IDENTIFICATION :

Chromosome analysis is otherwise known as karyotyping is one of the methods for distinguishing between species. Chromosome banding patterns can be used to distinguish human and mouse chromosomes and chromosome painting, i.e., using combinations of specific molecular probes to hybridize to individual chromosomes , adds further resolution and specificity to this technique. These probes identify individual chromosome pairs and are species specific. The availability of probes is limited to a few species at present, and most are either mouse or human, but chromosome painting is a good method for distinguishing between human and mouse chromosomes in potential cross-contaminations and interspecific hybrids. Isoenzyme electrophoresis , is also a good diagnostic test and is quicker than chromosomal analysis. A simple kit is available that makes this technique readily accessible . A combination of the two methods is often used and gives unambiguous results .

LINEAGE OR TISSUE MARKERS :

Individual organs are comprised of tissues, e.g., skin is made up of an outer epidermis and underlying dermis, and tissues, in turn, are made up of individual lineages, e.g., the dermis contains connective tissue fibrocytes, vascular endothelial cells and smooth muscle cells, and the mesenchymal cells of the dermal papillae, among others. Each cell type can be traced back, via a series of proliferating cell stages, to an originating stem cell , forming a treelike structure. Each

‘‘branch’’ of that ‘‘tree’’ can be regarded as a *lineage*, as in a basal cell of the epidermis following a differentiation path to a mature cornified keratinocyte. Some lineages, e.g., the myeloid lineage of hematopoietic differentiation, may branch into sublineages (neutrophilic, eosinophilic, and basophilic), so lineage marker expression is also influenced by *differentiation*, i.e., the position of the cell in the lineage differentiation pathway .Lineage markers are helpful in establishing the relationship of a particular cell line to its tissue of origin.

Cell surface antigens. These markers are particularly useful in sorting hematopoietic cells and have also been effective in discriminating epithelium from stroma with antibodies such as anti-EMA and anti-HMFG 1 and 2 and neuroectodermally derived cells (e.g., anti-A2B5) from cells derived from other germ layers.

Intermediate filament proteins. These are among the most widely used lineage or tissue markers. Glial fibrillary acidic protein (GFAP) for astrocytes and desmin for muscle are the most specific, whereas cytokeratin marks epithelial cells and mesothelium .Neurofilament protein marks neurons and some neuroendocrine cells ,although usually restricted to mesodermally derived cells *in vivo*, can appear in other cell types *in vitro*.

Differentiated products and functions. Hemoglobin for erythroid cells, myosin or tropomyosin for muscle, melanin for melanocytes, and serum albumin for hepatocytes are among the best examples of specific cell type markers, but, like all differentiation markers, they depend on the complete expression of the differentiated phenotype.

Enzymes. Three parameters are available in enzymic characterization: (1) the constitutive level (i.e., in the absence of inducers or repressors); (2) the response to inducers and repressors; and (3) isoenzyme polymorphisms.

TABLE 16.2. Enzymic Markers

Enzyme	Cell type	Inducer	Repressor
Glutamyl synthetase	Astroglia (brain)	Hydrocortisone	Glutamine
Tyrosine aminotransferase	Hepatocytes	Hydrocortisone	
Sucrase	Enterocytes	NaBt	
Alkaline phosphatase	Type II pneumocyte (in lung alveolus)	Dexamethasone, oncostatin, IL-6	TGF-β
Alkaline phosphatase	Enterocytes	Dexamethasone, NaBt	
Nonspecific esterase	Macrophages	PMA, Vitamin D_3	
Angiotensin-converting enzyme	Endothelium	Collagen, Matrigel	
Neuron-specific enolase	Neurons, neuroendocrine cells		
Tyrosinase	Melanocytes	cAMP	
DOPA-decarboxylase	Neurons, SCLC		
Creatine kinase MM	Muscle cells	IGF-II	FGF-1,2,7
Creatine kinase BB	Neurons, neuroendocrine cells, SCLC		

Unique Markers

Unique markers include specific chromosomal aberrations (e.g., deletions, translocations, polysomy); major histocom-patibility (MHC) group antigens (e.g., HLA in humans), which are highly polymorphic; and DNA fingerprinting or profiling.

CELL MORPHOLOGY :Observation of morphology is the simplest and most direct technique used to identify cells.

Microscopy : The inverted microscope is one of the most important tools in the tissue culture laboratory.

Staining :A polychromatic blood stain, such as Giemsa, provides a convenient method of preparing a stained culture. Giemsa stain is usually combined with May–Grunwald¨ stain when staining blood, but not when staining cultured cells. Alone, it stains the nucleus pink or magenta,

the nucleoli dark blue, and the cytoplasm pale gray-blue. It stains cells fixed in alcohol or formaldehyde, but will not work correctly unless the preparation is completely anhydrous.

CHROMOSOME CONTENT : Chromosome content or *karyotype* is one of the most characteristic and best-defined criteria for identifying cell lines and relating them to the species and sex from which they were derived. Chromosome analysis can also distinguish between normal and transformed cells ,because the chro-mosome number is more stable in normal cells.

CHROMOSOME BANDING : This group of techniques was devised to enable individual chromosome pairs to be identified when there is little morphological difference between them.

CHROMOSOME ANALYSIS : The following are methods by which the chromosome complement may be analyzed:

Chromosome Count. Count the chromosome number per spread for between 50 and 100 spreads. (The chromosomes need not be banded.) Closed-circuit television or a camera lucida attachment may help. You should attempt to count all of the mitoses that you see and classify them (a) by chromosome number or (b), if counting is impossible, as ‘‘near diploid uncountable’’ or ‘‘polyploid uncountable.’’ Plot the results as a histogram .

Karyotype. Digitally photograph about 10 or 20 good spreads of banded chromosomes.

CHROMOSOME PAINTING : With the advent of fluorescently labeled probes that bind to specific regions, and even specific genes, on chromosomes, it has become possible to locate genes, identify translocations, and determine the species of origin of chromosomes.

DNA CONTENT :DNA can be measured by propidium iodide fluorescence with a CCD camera or flow cytometry. Analysis of DNA content is particularly useful in the characterization of transformed cells that are often aneuploid and heteroploid.

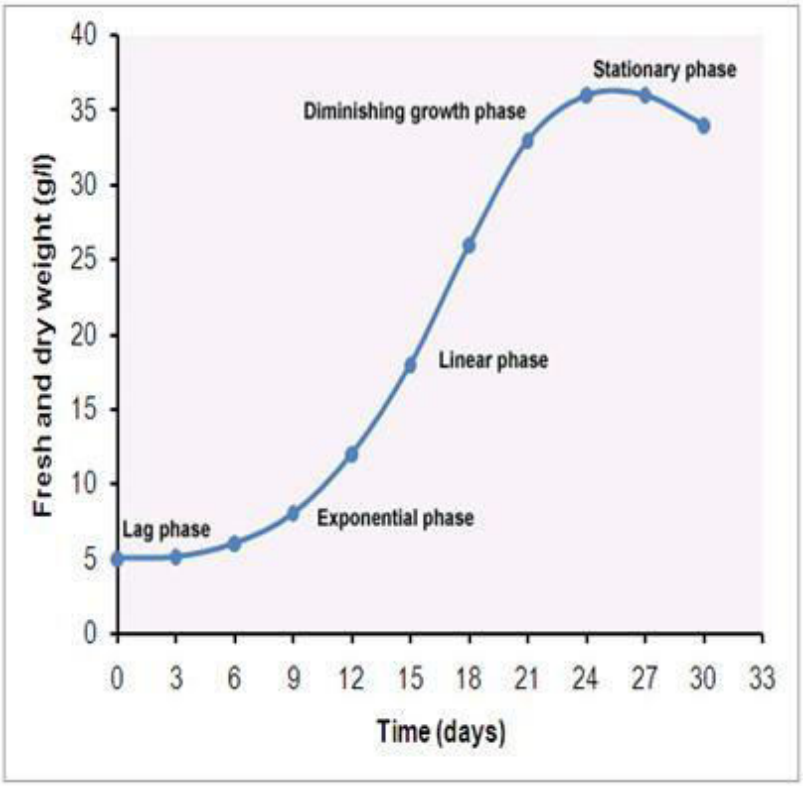
DNA HYBRIDIZATION : Hybridization of specific molecular probes to unique DNA sequences (Southern blotting) can provide information about species-specific regions, amplified regions of the DNA, or altered base sequences that are characteristic to that cell line.

DNA FINGERPRINTING :DNA contains regions known as satellite DNA that are apparently not transcribed. These regions are highly repetitive, and their lengths vary, with minisatellite

DNA having 1- to 30-kb repeats and microsatellite DNA having only 2–4 bases in repeating sequences. The functions of these regions are not fully understood; they may be purely structural or may provide a reservoir of potentially codable regions for genetic recombination in further evolution. Regardless of their function, however, these regions are not highly conserved, because they are not transcribed, and they give rise to regions of hypervariability. When the DNA is cut with specific endonucleases, specific sequences may be probed with cDNAs that hybridize to these hypervariable regions or they may be amplified by PCR with specific templates. The probes were originated by Jeffreys et al. . Electrophoresis reveals variations in fragment length in satellite DNA (restriction fragment length polymorphisms, RFLPs) that are specific to the individual from which the DNA was derived. When analyzed by polyacrylamide electrophoresis, each individual's DNA gives a specific hybridization pattern as revealed by autoradiography with radioactive or fluorescent probes. These patterns have come to be known as DNA fingerprints and are cell line specific, except if more than one cell line has been derived from one individual or if highly inbred donor animals have been used.

RNA AND PROTEIN EXPRESSION : Cells of a particular characteristic phenotype can be recognized by analysis of gene expression by Northern blotting [using radioactive, fluorescent, or luminescent probes.

KINETICS OF CELL GROWTH:


Fresh and dry weight (g/l)
Time (days)
Lag phase
Exponential phase
Linear phase
Diminishing growth phase
Stationary phase
0
5
10
15
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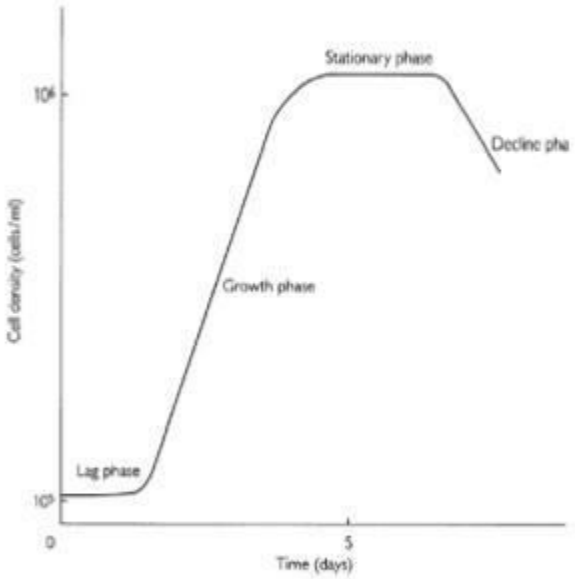
CELL GROWTH KINETICS



$$N = N_0 2^X$$

$$\log_{10} N = \log_{10} N_0 + X.\log_{10} 2$$

$$t_D = \frac{T}{X}$$

$$\mu = \frac{dN}{dt} \cdot \frac{1}{N}$$

$$\ln N = \ln N_o + \mu.t$$

$$\mu = \frac{\ln 2}{t_D} = \frac{0.6931}{t_D}$$

SCALE UP OF ANIMAL CELL CULTURES:

Modifying a laboratory procedure, so that it can be used on an industrial scale is called scaling up. Laboratory procedures are normally scaled up via intermediate models of increasing size. The larger the plant, the greater the running costs, as skilled people are required to monitor and maintain the machinery.The first pre-requisite for any large scale cell culture system and its scaling up is the establishment of a cell bank. Master cell banks (MCB) are first established and they are used to develop Master Working Cell Banks (MWCB). The MWCB should be sufficient to feed the production system at a particular scale for the predicted life of the product. The cell stability is an important criteria so MWCB needs to be repeatedly subcultured and each

generation should be checked for changes. A close attention should be paid to the volume of

cultured cells as the volume should be large enough to produce a product in amounts which is economically viable. The volume is maintained by a) increasing the culture volume, (b) by increasing the concentration of cells in a reactor by continuous perfusion of fresh medium, so that the cells keep on increasing in number without the dilution of the medium.

A fully automated bioreactor maintains the physicochemical and biological factors to optimum level and maintains the cells in suspension medium. The most suitable bioreactor used is a compact-loop bioreactor consisting of marine impellers. The animal cells unlike bacterial cells, grow very slowly. The main carbon and energy sources are glucose and glutamine. Lactate and ammonia are their metabolic products that affect growth and productivity of cells. So, the on-line monitoring of glucose, glutamate, and ammonia is carried out by on line flow injection analysis (FIA) using gas chromatography (GC), high performance liquid chromatography (HPLC) etc

.Cell cultures are used for obtaining useful products like bio-chemicals (interferon, interleukins, hormones, enzymes, antibodies, etc.) and virus vaccines (polio, mumps, measles, rabies, foot and mouth, rinderpest etc.).For these objectives, large scale cell cultures are essential; fermenters of 5,000 to 20,000 L are used for this purpose.

The scaling up of cell cultures may be done as follows:

(1) as monolayer cultures,

(2) as suspension cultures, or

(3) as immobilized cell systems.

For obvious reasons, scaling up of monolayer systems is more difficult than that of others.

1. Monolayer Culture:

Monolayer cultures are essential for anchorage dependent cells. Scaling up of such cultures is based on increasing the available surface area by using plates, spirals, ceramics and micro-carriers (most effective). The various culture vessels used are briefly described below.

Roux Bottle:

It is commonly used in laboratory, and is kept stationary so that only a portion of its internal surface is available for cell anchorage . Each bottle provides Ca. 175- 200 cm^2 surface area for cell attachment and occupies 750-1000 cm^3 space.

Roller Bottle:

This vessel permits a limited scale up as it is rocked or preferably, rolled so that its entire internal surface is available for anchorage. Several modifications of roller bottle further enhance the available surface,

e.g., (i) Spira-Cel (spiral polystyrene cartridge),

(ii) glass tube (roller bottle packed with a parallel cluster of small glass tubes separated by silicone spacer rings), and

(iii) extended surface area roller bottle (the bottle surface is corrugated enhancing the surface by a factor of two), etc.

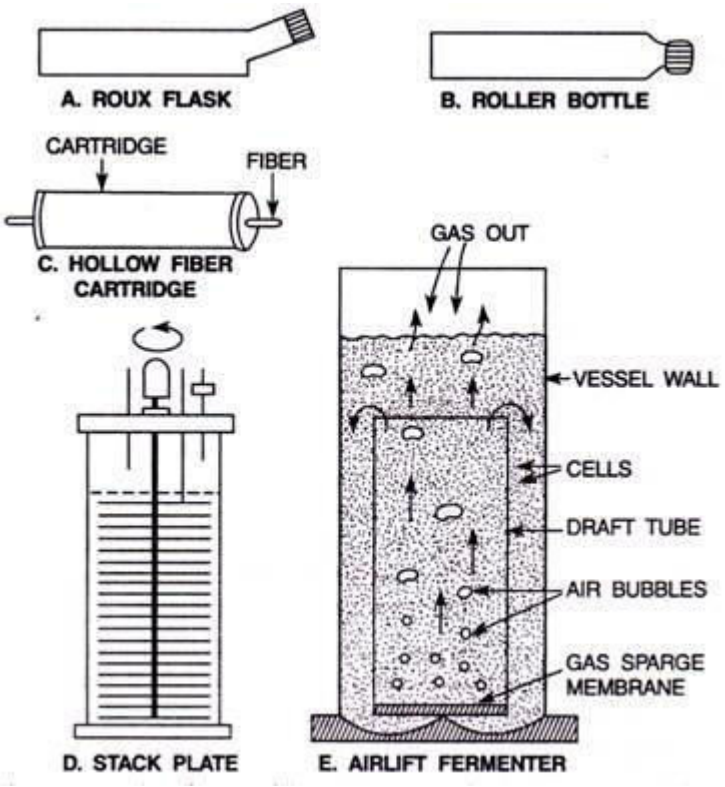


FIG. 5.3. A schematic representation of some of the various types of culture vessels used for scaling up of animal cell cultures. A. Roux flask, B. Roller bottle, C. Hollow fiber cartridge, D. Stack plate vessel, and E. Airlift fermentor.

Multitray Unit:

A standard unit has 10 chambers stacked on each other, which have interconnecting channels; this enables the various operations to be carried out in one go for all the chambers.

Each chamber has a surface area of 600 cm^2 and the total volume of the unit is 12.5 1. This polystyrene unit is disposable and gives good results similar to plastic flasks.

Synthetic Hollow Fibre Cartridge:

The fibres enclosed in a sealed cartridge provide a large surface area for cell attachment on the outside surface of fibres. The capillary fibres are made up of acrylic polymer, are 350 µm in diameter with 75 µm thick walls. The medium is pumped in through the fiber; it perfuses through the fiber walls and becomes available to the cells. The surface area available is very high (upto 30 cm^2/ml of medium volume). The system is mainly used for suspension cells, but is also suitable for cell anchorage if polysulphone type fiber is used.

Opticell Culture System:

It consists of a cylindrical ceramic cartridge in which 1 mm^2 channels run through the length of the unit, and perfusion loop to a reservoir is provided for environmental (medium, gas, etc.) control. It gives about 40 cm^2 surface area/ml of medium. It is suitable for virus, cell surface antigen and monoclonal antibody production, and for both suspension and monolayer cell cultures.

Plastic Film:

Teflon (fluoroethylenepropylene copolymer) is biologically inert and highly permeable to gas. Teflon bags (5 x 30 cm) filled with cells and medium (2-10 mm deep) serve as good culture vessels; cells attach to the inside surface of bags. Alternatively, teflon tubes are wrapped round a reel with a spacer and the medium is pumped through the tube; cells grow on the inside surface of tube (a culture vessel called stericell is available).

Heli-Cell vessels:

These vessels are packed with polystyrene ribbons (3 mm x 5-10 mm x 100 µm) that are twisted in helical shape. The medium is pumped through the vessel, the helical shape of ribbons ensuring good circulation; the cells adhere to the ribbon surfaces.

All the culture vessels, in addition to the increased surface area due to the vessel design, allow further scaling up by the use of multiple units of the vessels. In contrast, the following three culture systems allow scaling up in a single unit by increasing the vessel volume. In addition,

they make the monolayer culture system considerably similar to suspension cultures.

Bead Bed Reactors:

These reactors are packed with 3-5 mm glass beads (which provide the surface for cell attachment) and the medium is pumped either up or down the bead column. Use of 5 mm beads gives better cell yields than that of 3 mm beads.

Heterogeneous Reactors:

These reactors contain circular glass or stainless steel plates stacked 5-7 mm apart and fitted to a central shaft . Either an airlift pump is used for mixing or the shaft is rotated either vertically or horizontally. The chief disadvantage of the system is very low ratio of surface area to medium volume (1-2 cm^2/ml).

Microcarrier cultures:

These systems use 90-300 pm dia particles as substrate for cell attachment. Initially, Dextran beads (Sephadex A-50) were used by Van Wezel in 1967; these were not entirely satisfactory due to the unsuitable charge of beads and possibly due to toxic effects.

The microcarriers available for use at present range from Dextran, polystyrene, polyacrolein, glass, poly- acrylamide, silica, DEAE sephadex, cellulose, gelatin to collagen; the specific gravity of microcarriers ranges from 1.02 to 1.05. Microcarriers greatly increase the surface area for cell attachment per unit medium volume, which can be upto 90,000 cm^2/ L depending on the size and density of the beads.

Microcarrier cultures are initiated by harvesting cells from 3 L of a logarithmic phase (log phase) culture and inoculating them in 1 L of fresh medium to which 2-3 g/L of microcarriers is then added. The culture is stirred at 15-25 rpm (revolutions per minute) for 3-8 hours. During this period, cells attach to microcarrier beads and later grow as a monolayer.

The volume of culture is slowly increased to 3 1 and stirring is enhanced to the normal rates (20-100 rpm). As the cells grow, the beads become heavier and need to be agitated at higher speeds. The medium needs to be changed every 3 days. Samples of beads can be drawn for observations on cell morphology, growth and number.

Use of microcarriers permits the handling of monolayer systems as suspension cultures. However, cells do not grow to the same degree as they do in stationary cultures. Harvesting of

cells from microcarrier beads in rather simple.Stirring is stopped, the medium is drained off, the beads are washed in buffer, treated with trypsin or some other suitable enzyme, the culture is shaken at 75-125 rpm for 20-30 min, stirring is stopped for 2 min and the supernatant is poured and collected.

Alternatively, the beads may be dissolved where possible, e.g., gelatin beads are dissolved by trypsin, collagen-coated beads are treated with collagenase and dextranase is used for dextran beads; these treatments leave the cells free, which are collected.

Scaling up of microcarrier cultures can be done either by increasing the concentration of beads or by enlarging the culture vessel. When high microcarrier concentrations are used, medium perfusion becomes necessary, and efficient filters must be used to allow medium withdrawal without cells and microcarriers. The oxygen supply is problematic; it can be based on surface aeration, increased perfusion rate of fully aerated medium, and sparging into the filter compartment.

2. Suspension Cultures:

In scaling up, both chemical (O_2, pH, medium constituents and removal of wastes) and physical (the configuration of bioreactor and power supplied to the reactor) factors have to be optimised for good results. The medium must be suitably stirred to keep the cells in suspension and to make the culture homogeneous; it becomes increasingly difficult with the scaling up.

Various types of stirrers range from simple magnetic stirrers, flat blade turbine impellers, to marine impellers, to those using pneumatic energy, e.g., airlift fermenter, and those using hydraulic energy, e.g., medium perfusion. Improved mixing can be obtained by changing the design of stirrer paddle or by using multiple impellers.

The objective of stirring is to achieve good mixing without causing damage to the cells. Vibro-mixer achieves stirring by vertical reciprocating motion of 0.1-S mm at a frequency of 50 cycles/sec of a mixing disc fixed horizontally to the agitator shaft. These stirrers cause random mixing, less foaming and lower shear forces.

It is important to supply sufficient O_2 without damaging the cells. Mean 0_2 utilization rate by cells is about 6 mg $O_2/10^6$ cells/hour. But O_2 is only sparingly soluble in culture medium; the oxygen transfer rate (OTR) from gas phase into medium is about 17 μg /cm^2/hr.

Therefore, surface aeration can support about 50 x 10^6 cells in a 1 L culture vessel. When the medium depth in a culture is above 5 mm (especially, above 5 cm), aeration with a mixture of $C0_2$ and air becomes necessary to maintain adequate gas exchange.

Efficient aeration is achieved by bubbling air through the medium (sparging), but this may damage animal cells due to the high surface energy of the bubble and on the cell membrane. The damage can be reduced by using larger bubbles, lower gassing rates and by adding non-nutritional supplements like Pluronic F-6B (polyglycol), sodium carboxymethyl cellulose and polyvinylphyrrolidone (these protect cells from damage due to shear forces and bubbles, respectively). Silicone tubing (highly gas permeable) can be arranged inside the culture vessel (2-5 cm tubing of 30 m length for a 1000 L culture) and air is passed though the tube; however it is inconvenient to use.

Aeration may be achieved by medium perfusion, in which medium is continuously taken from culture vessel, passed through an oxygenation chamber and returned to the culture. The cells are removed from the medium taken for perfusion so that the medium can be suitably altered, e.g., for pH control. Perfusion is used with glass bead and, more particularly, with micro carrier systems.

Where considered safe and desirable, O_2 supply in the culture vessel can be enhanced from the normal 21% to a higher value and the air pressure can be increased by 1 atmosphere. This increases the O_2 solubility and diffusion rates in the medium, but there is a risk of O_2 toxicity.

The reactors used for large scale suspension cultures are of 3 main types:

(1) Stirred tank bioreactors,

(2) Continuous flow reactors, and

(3) Airlift fermenters.

Stirred Tank Bioreactors:

These are glass (smaller vessels) or stainless steel (larger volumes) vessels of 1-1,000 1 or even 8,000 1 (Namalva cells grown for interferon; but in practice their maximum size is 20 1 since larger vessels are difficult to handle, autoclave and to agitate the culture effectively).

These are closed systems with fixed volumes and are usually agitated with motor-driven stirrers with considerable variation' in design details, e.g., water jacket in place of heater type

temperature control, curved bottom for better mixing at low speeds, mirror internal finishes to reduce cell damage, etc. Many heteroploid cell lines can be grown in such vessels.

The needs for research biochemical from cells are met from 2-50 1 reactors, while large scale reactors are mainly used for growing hybridoma cells for the production of monoclonal antibodies although their yields from cultured cells is only 1-2% of those obtained by passaging the cells through peritoneal cavity of mice.

Continuous-Flow Cultures:

These culture systems are either of chemostat or turbidostat type. In both the types, cultures begin as a batch culture. In a chemostat type, inoculated cells grow to the maximum density when some nutrient, e.g., a vitamin, becomes growth limiting. Fresh medium is added after 24-48 hours of growth, at a constant rate (usually lower than the maximum growth rate of culture) and at an equal rate the culture is withdrawn.

When the rate of growth equals the rate of cell withdrawal, the cultures are in a 'steady state', and both the cell density and medium composition remain constant. One of the constituents of the medium is used at a lower concentration to make it growth-limiting. However, chemostat is the least efficient or controllable at the cell's maximum growth rate hence the steady-state growth rates in them are much lower than the maximum.

In contrast, in a turbidostat cells grow to achieve a predecided density (measured as turbidity using a photoelectric cell). At this point, a fixed volume of culture is withdrawn and the same volume of fresh normal (not having a growth-limiting factor) medium is added; this lowers the cell density or turbidity of the culture. Cells keep growing, and once the culture reaches the preset density the fixed volume of culture is replaced by fresh medium. This system works really well when the growth rate of the culture is close to the maximum for the cell line.

The continuous-flow cultures provide a continuous source of cells, and are suitable for product generation, e.g., for the production of viruses and interferons. It is often necessary to use a two- stage system in which the first stage supports cell growth, while the second stage promotes product generation.

Airlift Fermenters:

Cultures in such vessels are both aerated and agitated by air (5% CO_2 in air) bubbles introduced at the bottom of vessels . The vessel has an inner draft tube through which the air

bubbles and the aerated medium rise since aerated medium is lighter than non- aerated one; this results in mixing of the culture as well as aeration.

The air bubbles lift to the top of the medium and the air passes out through an outlet. The cells and the medium that lift out of the draft tube move down outside the tube and are recalculated. O_2 supply is quite efficient but scaling up presents certain problems. Fermenters of 2-90 L are commercially available, but 20,000 L fermenters are being used by biotechnology industries.

3. Immobilized Cultures:

Cultures based on immobilized cells offer the following several advantages: (1) higher cell densities (50-200 x 10^{-6} cells/ml), (2) stability and longevity of cultures, (3) suitability for both suspension and monolayer cultures, (4) protection of the cells from shear forces due to medium flow (in case of many systems), and (5) less dependence of cells at higher densities on external supply of growth factors, which saves culture cost. There are the following two basic approaches to cell immobilization: (1) immurement

(2) entrapment.

Immurement Cultures:

In such cultures, cells are confined within a medium permeable barrier. Hollow fibers packed in a cartridge are one such system. The medium is circulated through the fiber, while cells in suspension are present in the cartridge outside the fiber.

This is extremely effective for scales upto 1 1 and gives cell densities of 1-2 X 10^8cells/ml; sophisticated units can yield upto 40 g monoclonal antibodies/month. Membranes permitting medium and gas diffusion are also used to develop bioreactors of this type; both small scale and large scale versions of membrane bioreactors are available commercially.

The cells may be encapsulated in a polymeric matrix by adsorption, covalent bonding, cross- linking or entrapment; the materials used as matrix are gelatin, polylysine, alginate and agarose. This approach (1) effectively protects cells from mechanical damage in large fermenters, and (2) allows production of hormones, antibodies, immunochemicals and enzymes over much longer periods than is possible in suspension cultures. (3) The medium diffuses freely into the matrix and into the cells, while cell products move out into the medium.

For production of larger molecules like monoclonal antibodies, agarose in a suspension

of paraffin oil is preferable to alginate since the latter does not allow "diffusion of such products out of the alginate beads. Reactors of upto 3 1 are available commercially.

Entrapment Cultures:

In this approach, cells are held within an open matrix through which the medium flows freely. An example is the Opticell, in which the cells are entrapped within the porous ceramic walls of the unit.

Opticell units of upto 210 m^2 surface area are available, which can yield upto 50 g monoclonal antibodies per day. The cells can also be enmeshed in cellulose fibres, e.g., DEAE, TLC, QAE, and TEAE. These fibers are autoclaved and washed as prescribed and added in a spinner/stirred bioreactor at a concentration of 3 g/1.

Porous macrocarriers:

Porous macrocarriers are small (170 pm-6,000 pm) beads of gelatin, collagen, glass or cellulose, which have a network of interconnecting pores. These provide a tremendous enhancement in surface area/volume ratio, permit efficient diffusion of medium and product, are suitable for scaling up, and are equally useful for suspension and monolayer cultures.These can be arranged as fixed bed or fluidized bed reactors or used in stirred bioreactors. It is expected that future developments will make the immobilized cell systems the most dominant production systems.

Adherent Cell Culture	Suspension Cell Culture
Appropriate for most cell types, including primary cultures	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic)
Requires periodic passaging, but allows easy visual inspection under inverted microscope	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to

	stimulate growth
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically	Does not require enzymatic or mechanical dissociation
Growth is limited by surface area, which may limit product yields	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Requires tissue-culture treated vessel	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exchange
Used for cytology, harvesting products continuously, and many research applications	Used for bulk protein production, batch harvesting, and many research applications

The Rotary Cell Culture System (RCCS):It is a device designed to grow three-dimensional cell clusters in microgravity.

NASA BIOREACTOR:

The NASA Bioreactor was developed by NASA to simulate the weightless environment of space by putting cells in a growth medium that constantly rotates and keeps the cells in endless free-fall.Culturing cells means putting some small number into nutrient media in a dish

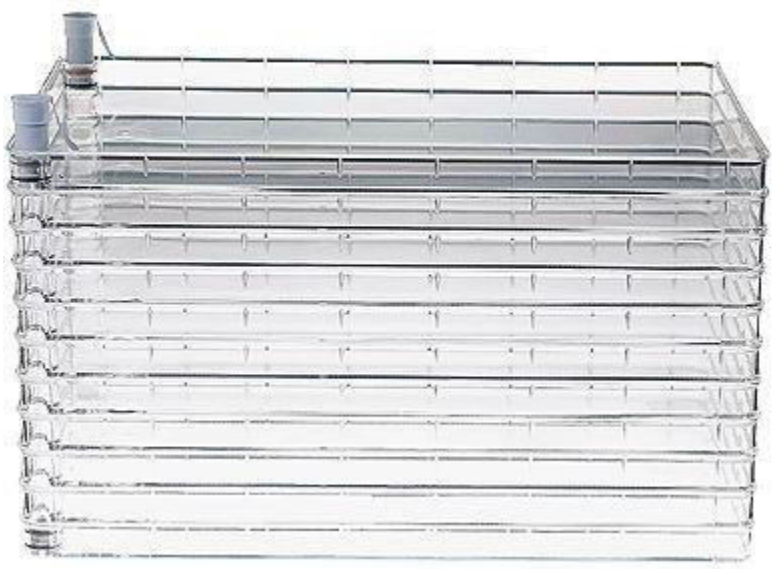
or a tube and letting them grow. However, this kind of approach does not provide the culture environment that supports tissue assemblies. Without a proper 3-D assembly, epithelial cells (the basic cells that differentiate tissue into specific organ functions) lack the proper clues for growing into the variety of cells that make up a particular tissue.

In a rotating Bioreactor, the cells can be fooled into thinking they are in a body. With a plastic lattice to help direct their growth, cells can be encouraged to grow in predefined shapes.

NUNC CELL FACTORY:

- Closed system with sterile fluid path
- Store or transport cells
- Excellent imaging properties
- Freeze and thaw directly in OptiCell

The Nunc OptiCell cell culture system has very stable growth conditions, as O2 and CO2 are efficiently diffused through the thin film. OptiCell products have low space requirements (compared to tissue culture flasks) and a low media consumption because of the large growth area-to-volume ratio. Besides growth, imaging, transport and storage of cell cultures, OptiCell cell culture system can also be used for biomagnetic cell separation and has proven itself as excellent for hybridoma antibody production and transfection studies.



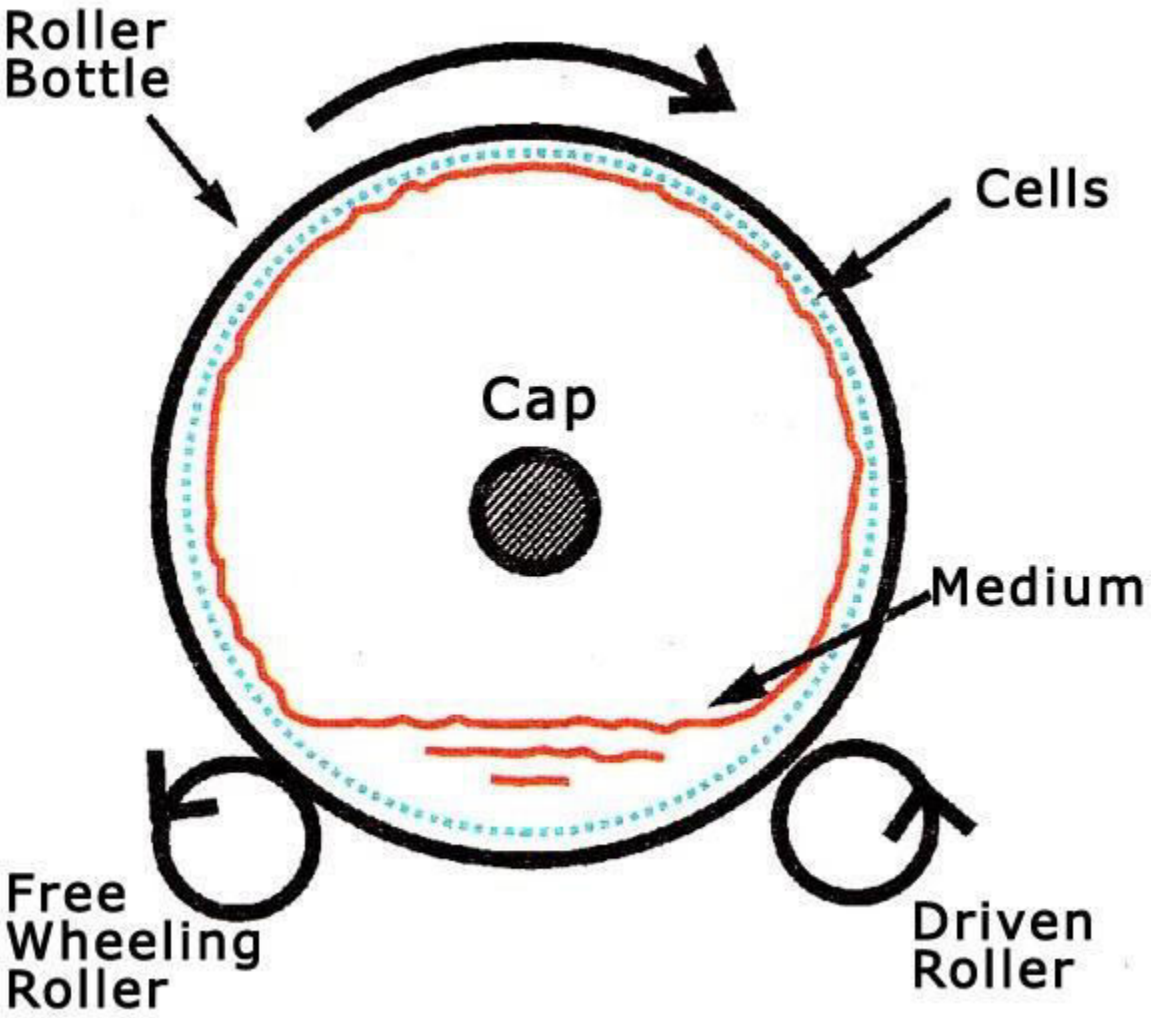
Nunc Cell Factory

In batch cultures, mainly Roller Bottles with Micro Carrier Beads (for adherent cells) and spinner flasks (for suspension cultures) are used in Scale-up of animal cell culture process.

Roller Bottles:

The Roller bottles provide total curved surface area of the micro carrier beads for growth. The continuous rotation of the bottles in the CO2 incubators helps to provide medium to the entire cell monolayer in culture.The roller bottles are well attached inside a specialized CO2 incubators. The attachments rotate the bottles along the long axis which helps to expose the entire cell monolayer to the medium during the one full rotation. This system has the advantage over the static monolayer culture: (a) it provides increase in the surface area, (b) provides constant gentle agitation of the medium, (c) provides increased ratio of surface area of medium to its volume, which allows gas exchange at an increased rate through the thin film of the medium over the cells. Typically, a surface area of 750-1500 cm2 with 200-500 ml medium will yield 1-2x108cells.

DIAGRAM SHOWING THE ROLLER BOTTLE CELL CULTURE



Micro Carrier Beads:

Micro carrier beads are small spherical particles with diameter 90-300 micrometers, made up of dextran or glass. Micro Carrier beads, increase the number of adherent cells per flask. These dextran or glass-based beads come in a range of densities and sizes. The cells grow at a very high density which rapidly exhausts the medium and therefore the medium has to be replaced for the optimum cell growth. At the recommended concentration when the microcarriers are suspended they provide 0.24 m2 area for every 100 ml of culture flask.

Spinner cultures:

The spinner flask, was originally developed to provide the gentle stirring of microcarriers but are now used for scaling up the production of suspension cells. The flat surface glass flask is fitted with a Teflon paddle that continuously turns and agitates the medium. This stirring of the medium improves gas exchange in the cells in culture. The spinner flask used at commercial scale consists of one or more side arms for taking out samples and decantation as well.

Primary Cells Immortalized Cell Lines
Lifespan:
Limited, resembles tissue characteristics -PC Infinite, loses tissue characteristics- ICL
Closer to an in vivo model:
Yes, isolated directly from the tissue-PC No, clonally selected over time-ICL
Reduces animal testing costs:
Yes, used in advanced cell culture models to refine experiments-PC Limited ability to develop biologically relevant complex in vitro models-ICL
Mutations/Modifications:
Low-PC High-ICL
Authentication required before use:
No, if bought commercially-PC

Yes, mandated by many government institutes and scientific journals -ICL

Availability of donor characteristics:

Yes-PC

No-ICL

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SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – III– ANIMAL BIOTECHNOLOGY – SBT1305

UNIT III ANIMAL TRANSGENESIS

Transgenic models: Methods of producing transgenic mice,Chicken & Livestock -Embryonic stem cell, pronucleus method-random and target gene insertion, knockout and knockin mice.

METHODS OF PRODUCING TRANSGENIC MICE:

Transgenic Animals:

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it

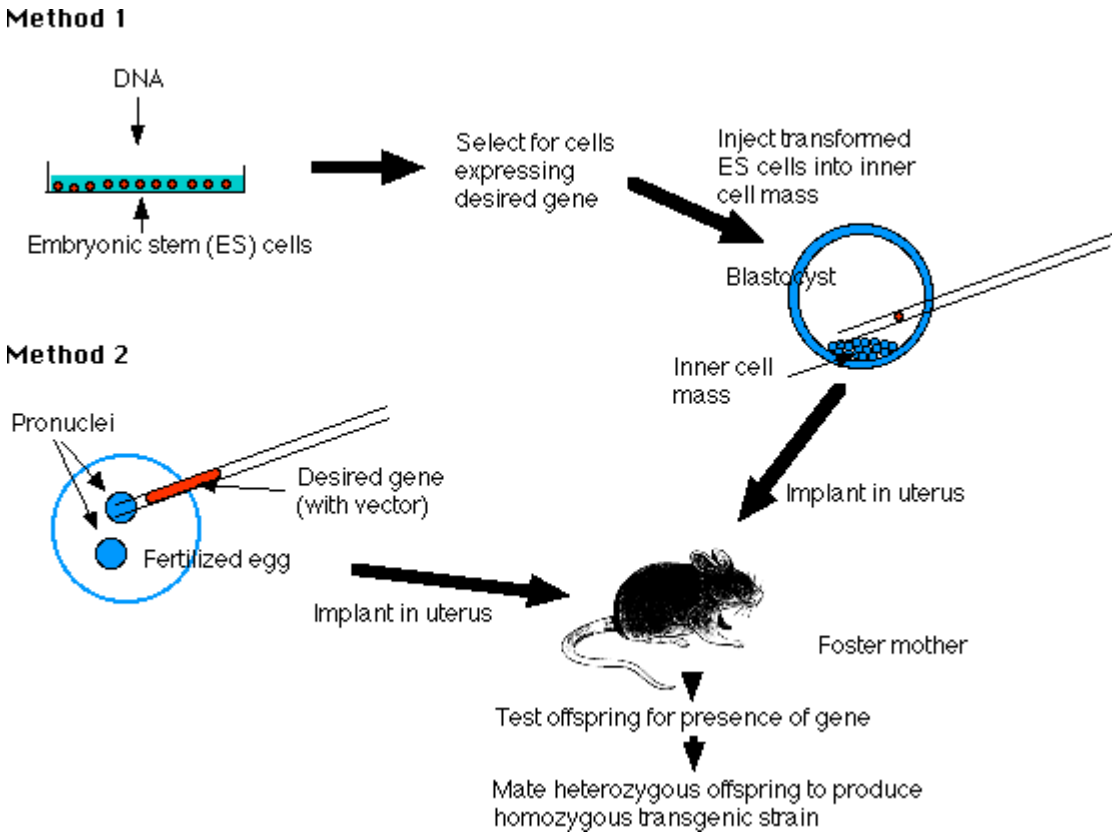
- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.
- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs.

Two methods of producing transgenic mice are widely used:

- **Embryonic stem cells** (**ES** cells) growing in tissue culture with the desired DNA;
- injecting the desired gene into the **pronucleus** of a fertilized mouse egg.

THE EMBRYONIC STEM CELL METHOD : (METHOD "1")

Embryonic stem cells (**ES** cells) are harvested from the **inner cell mass** (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal,**including its gametes**.



1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

- the gene you desire (e.g., the insulin gene);
- **vector** DNA to enable the molecules to be inserted into host DNA molecules;
- promoter and enhancer sequences to enable the gene to be expressed by host cells.

2. Transform ES cells in culture: Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells.

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

- Prepare a **pseudopregnant** mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they **implant** successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10–20% will have it, and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1 in 4 that will be **homozygous** for the transgene.
- Mating these will found the transgenic strain.

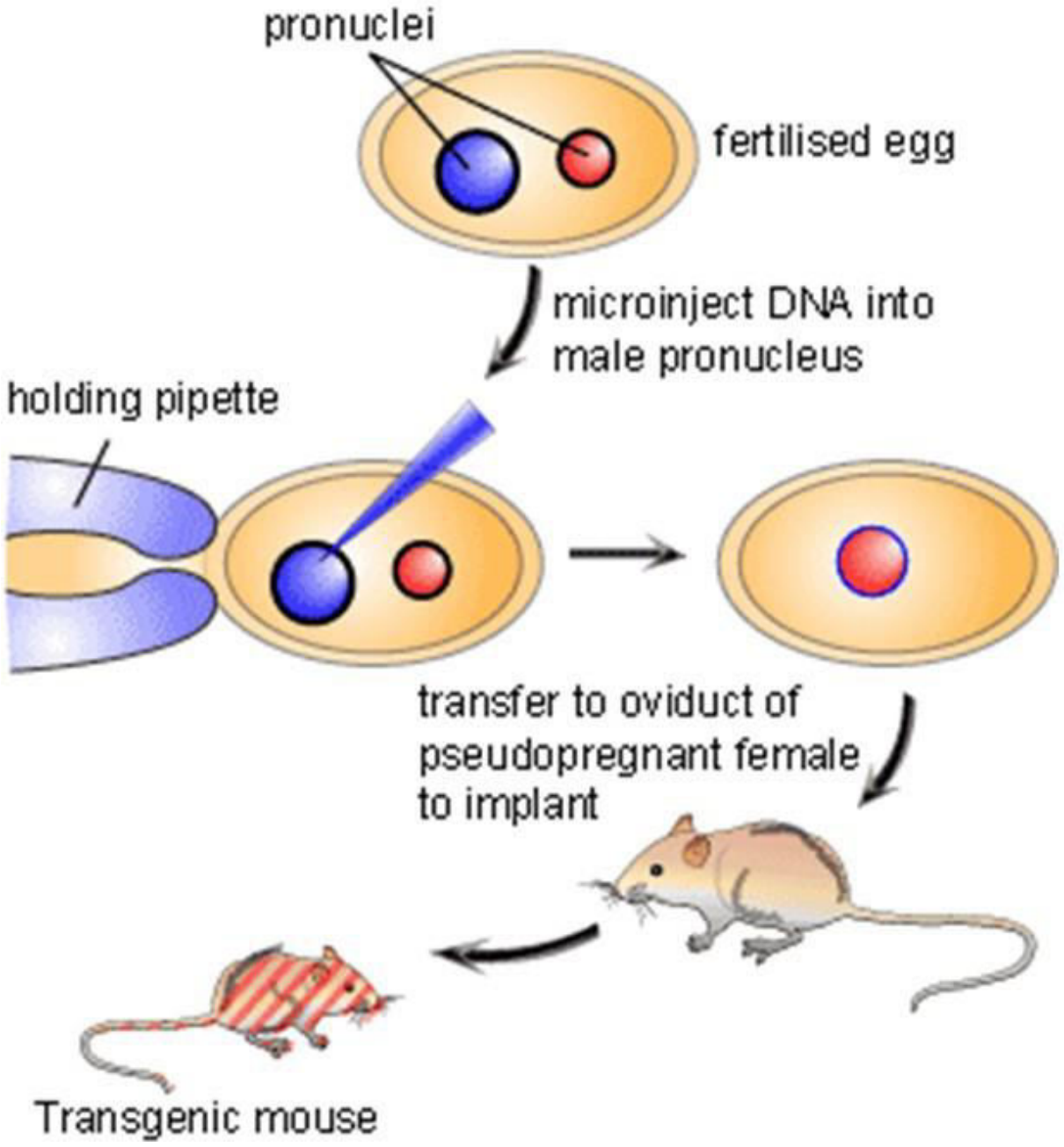
THE PRONUCLEUS METHOD (METHOD "2")

1. Prepare your DNA as in Method 1

2. Transform fertilized eggs

- Harvest freshly fertilized eggs before the sperm head has become a pronucleus.
- Inject the male pronucleus with your DNA.
- When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.

3. Implant the embryos in a pseudopregnant foster mother and proceed as in Method 1.


pronuclei
fertilised egg
microinject DNA into
male pronucleus
holding pipette
transfer to oviduct of
pseudopregnant female
to implant
Transgenic mouse

TRANSGENESIS- METHODS OF GENE TRANSFER:

Transgenesis is the process of introducing an exogenous gene — called a transgene — into a living organism so that the organism will exhibit a new property and transmit that property to its offspring. Transgenesis can be facilitated by liposomes, plasmid vectors, viral vectors, pronuclear injection, protoplast fusion, and ballistic DNA injection.

Transgenic organisms are able to express foreign genes because the genetic code is similar for all organisms. This means that a specific DNA sequence will code for the same protein in all organisms. Due to this similarity in protein sequence, scientists can cut DNA at these common protein points and add other genes. An example of this is the "super mice" of the 1980s. These mice were able to produce the human protein tPA to treat blood clots.

Transgenic Animals: Objectives of Gene Transfer with Different Transfection Methods!

A transgenic animal contains in its genome, a gene or genes introduced by one or the other technique of transfection.

The gene introduced by transfection is called a transgene. In animals transfection specifies the introduction of a DNA segment, either naked or integrated into a vector, into an animal cell.

Objectives of Gene Transfer:

1. Genetic modification of animals may be aimed at improving their milk, meat, wool, etc. production.

2. Genes have been transferred into animals to obtain a large scale production of the proteins encoded by these genes in the milk, urine or blood of such animals.

3. A special case of gene transfer aims at alleviating or even eliminating the symptoms and consequent miseries of genetic diseases. In this approach, normal and functional copies of the defective gene are introduced into the patient (gene therapy).

4. Specific transgenic animal strains or lines are created to fulfill specialized experimental and/or biomedical needs.

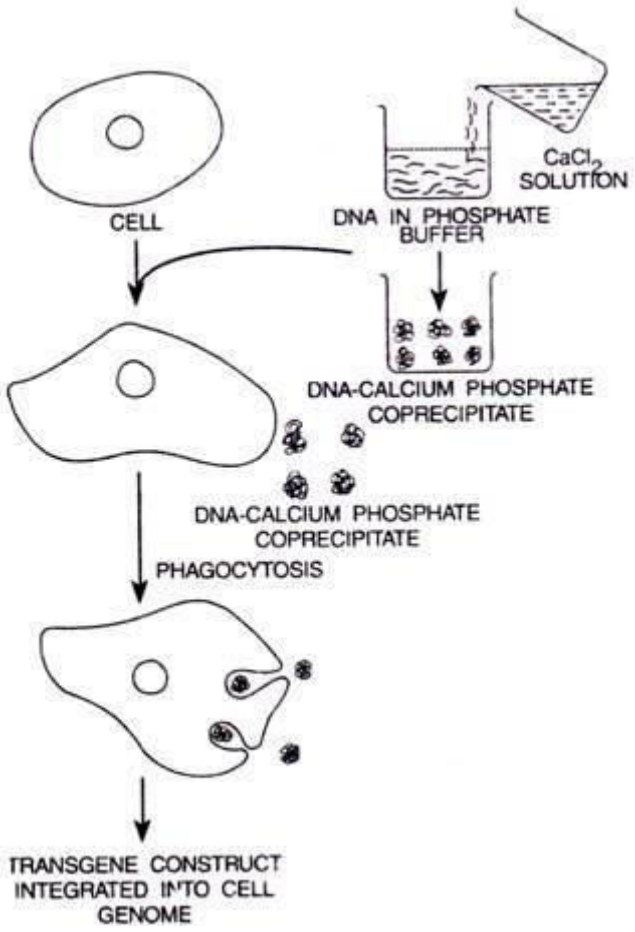
Transfection Methods:

Calcium Phosphate Precipitation:

In this approach, the DNA preparation to be used for transfection is first dissolved in a phosphate buffer. Calcium chloride solution is then added to the DNA solution; this leads to the formation of insoluble calcium phosphate which co-precipitates with the DNA. The calcium phosphate-DNA precipitate is added to the cells to be transfected.

The precipitate particles are taken in by the cells by phagocytosis. Initially, 1-2% of the cells were transfected by this approach. But the procedure has now been modified to obtain transfection of upto 20% of the cells. In a small proportion of the transfected cells, the DNA becomes integrated into the cell genome producing stable or permanent transfection.

This general approach can be applied to virtually all mammalian cells, and a very large number of cells can be treated with little effort. But many cell lines do not like the calcium phosphate precipitate adhering to their surfaces or to their substrate.



DEAE-Dextran-Mediated Transfection:

DEAE-dextran (dimethylaminoethyl-dextran) is water soluble and polycationic, i.e., has a multiple positive charge. It is added to the transfection solution containing the DNA. In some unknown way, DEAE-dextran brings about DNA uptake by the cells through endocytosis.

Possibly its interaction with the negatively charged DNA molecules and with the components of cell surface plays an important role. This procedure is highly suited for transient transfection used for various molecular biology studies, particularly using COS cell lines. However, again for some unknown reason, it is not efficient in producing stable transfection.

Lipofection:

The delivery of DNA into cells using liposomes is called lipofection. Liposomes are small vesicles prepared from a suitable lipid. Initially, nonionic lipids were used for preparing liposomes so that DNA had to be introduced within the vesicles following specific encapsidation procedures.

The use of cationic lipids for the construction of liposomes is a distinct advantages as DNA spontaneously and efficiently complexes with these liposomes making encapsidation procedures unnecessary. The cationic liposomes have a single lipid bilayer membrane (unilamellar), and they bind to the cells efficiently. Probably they fuse with the plasma membrane and thereby deliver the DNA (complexed with them) into the cells, which brings about transfection.

Lipofection is the method of choice for transfection of mammalian cells in vitro. It has also been used to deliver DNA into live animals by direct injection or intravenous injection. Cationic liposomes have been used in intravenous or intratracheal injection in mice for the expression of marker genes in lungs. Targeted delivery has also been demonstrated by incorporating specific ligand proteins into the liposome membranes.

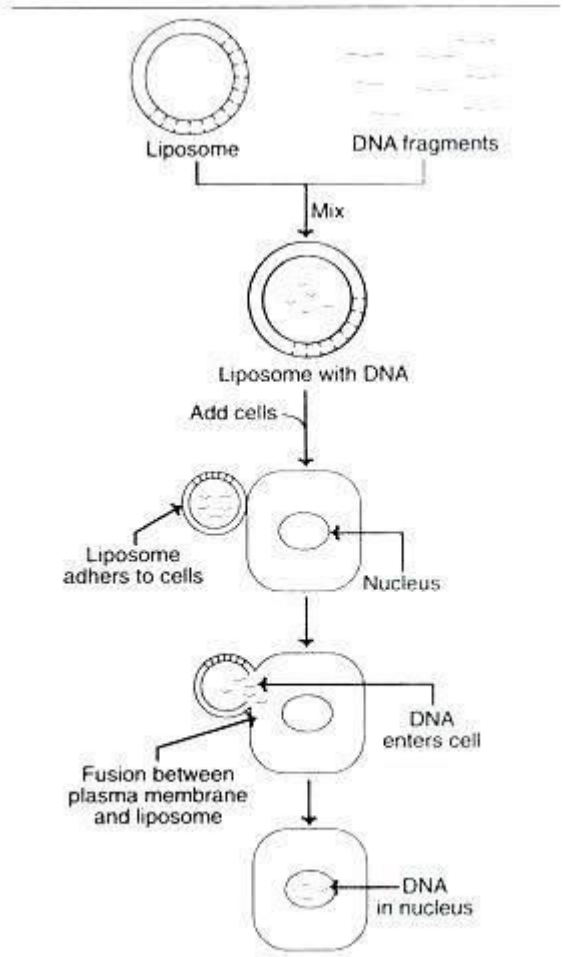
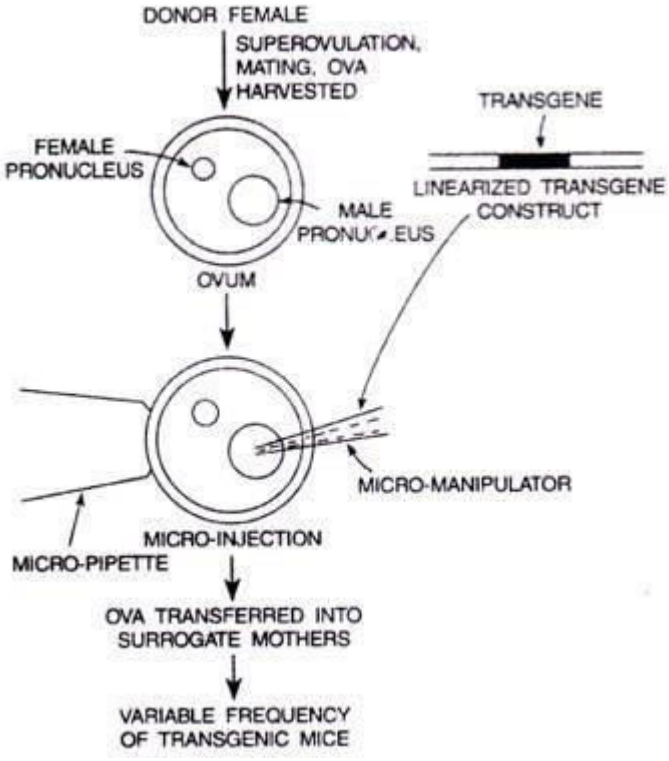


Fig. 6.12 : Liposome-mediated gene transfer
(Note : For clarity, the native cell DNA is not shown).

Microinjection:

In this method, DNA solution is injected directly into the nucleus of a cell or into the male pronucleus of a fertilized one to two-cell ovum. Typically, a microinjection assembly consists of a low power stereoscopic dissecting microscope (to view the ovum and the entire process) and two micromanipulators, one for a glass micropipette to hold the ovum by partial suction and the other for a glass injection needle to introduce the DNA into the male pronucleus. The male pronucleus is much larger than the female pronucleus of fertilized mammalian ova. However in fish ova, the DNA is injected into the egg cytoplasm.

The general procedure for microinjection is as follows: Donor females are induced to super-ovulate using appropriate hormone treatments. The superovulated females are then mated with fertile males, and large numbers of fertilized one or two-cell ova/embryos are collected surgically. Alternatively, unfertilized ova are collected from super-ovulated females; the ova are then fertilized in vitro. The transgene construct is prepared in a buffer solution and is injected into the male pronuctei of fertilized eggs using a microinjection assembly.



PARTICLE GUN/BIOLISTICS:

A gene gun or a biolistic particle delivery system, originally designed for plant transformation, is a device for injecting cells with genetic information; the inserted genetic material are termed transgenes. The payload is an elemental particle of aheavy metal coated with plasmid DNA. This technique is often simply referred to as bioballistics or biolistics.

This device is able to transform almost any type of cell, including plants, and is not limited to genetic material of the nucleus: it can also transform organelles, including plastids.

Biolistic construct design:

A construct is a piece of DNA inserted into the target's genome, including parts that are intended to be removed later.[5] All biolistic transformations require a construct to proceed and while there is great variation among biolistic constructs, they can be broadly sorted into two categories: those which are designed to transform eukaryotic nuclei, and those designed to transform prokaryotic-type genomes such as mitochondria, plasmids or plastids.[5]

Those meant to transform prokaryotic genomes generally have the gene or genes of interest, at least one promoter and terminator sequence, and a reporter gene; which is a gene used to ease detection or removal of those cells which didn't integrate the construct into their DNA.[5] These genes may each have their own promoter and terminator, or be grouped to produce multiple gene products from one transcript, in which case binding sites for translational machinery should be placed between each to ensure maximum translational efficiency. In any case the entire construct is flanked by regions called border sequences which are similar in sequence to locations within the genome, this allows the construct to target itself to a specific point in the existing genome.[5]

Constructs meant for integration into a eukaryotic nucleus follow a similar pattern except that: the construct contains no border sequences because the sequence rearrangement that prokaryotic constructs rely on rarely occurs in eukaryotes; and each gene contained within the construct must be expressed by its own copy of a promoter and terminator sequence.[5]

Though the above designs are generally followed, there are exceptions. For example, the construct might include a Cre-Lox system to selectively remove inserted genes; or a prokaryotic construct may insert itself downstream of a promoter, allowing the inserted genes to be governed by a promoter already in place and eliminating the need for one to be included in the construct.

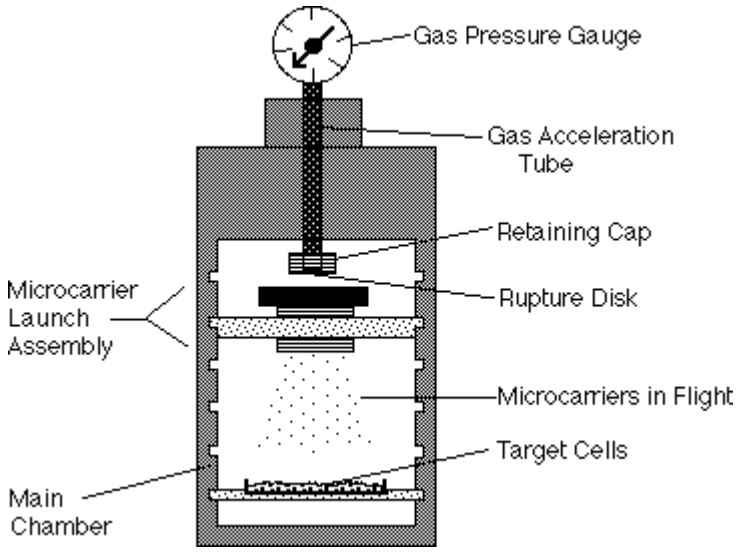
APPLICATIONS:

Gene guns have also been used to deliver DNA vaccines.

The delivery of plasmids into rat neurons through the use of a gene gun, specifically DRG neurons, is also used as a pharmacological precursor in studying the effects of neurodegenerative diseases such as Alzheimer's disease.

The gene gun has become a common tool for labeling subsets of cells in cultured tissue. In addition to being able to transfect cells with DNA plasmids coding for fluorescent proteins, the gene gun can be adapted to deliver a wide variety of vital dyes to cells.[6]

Gene gun bombardment has also been used to transform *Caenorhabditis elegans*, as an alternative to microinjection.



Electroporation:

In this approach, transfection mixture containing cells and DNA is exposed for a very brief period (few milliseconds) to a very high voltage gradient (e.g., 4,000-8,000 V/cm). Electroporation is generally done at room temperature, but the cells are subsequently kept on ice to allow the membrane pores to remain open for a longer duration.

This induces transient pores in the cell membranes through which DNA seems to enter the cells. Treatment of cells with colcemid before they are electroporated increases the frequency of

transfection. This is most likely due to the arrest of cells at metaphase and the associated absence of nuclear envelope or to an unusual permeability of the plasma membranes.

Linearized DNA is far more efficient in transfection than circular supercoiled DNA possibly because of a higher frequency of integration of linear DNA into the genome. Generally, DNA integration occurs in a low copy number, while chemical methods usually lead to multiple copy integration per genome. Electroporation technique has a general applicability, and many animal cell types that could not be transfected by other approaches were successfully transfected by this approach.

A single cell electroporation microarray based on a silicon chip of 1 cm^2 has been developed. The chip has 60 circular, cell-sized (20 pm diameter) microelectrodes each one of which is contacted by a dedicated neutral line. The silicon chip is placed in a plastic chamber designed to hold culture medium. The cells are cultured on the chip surface, and they can be individually electroporated by a PC-driven control system. The DNA can be delivered to several preselected individual cells within the same culture, at arbitrarily chosen time points, and even sequentially to the same cell.

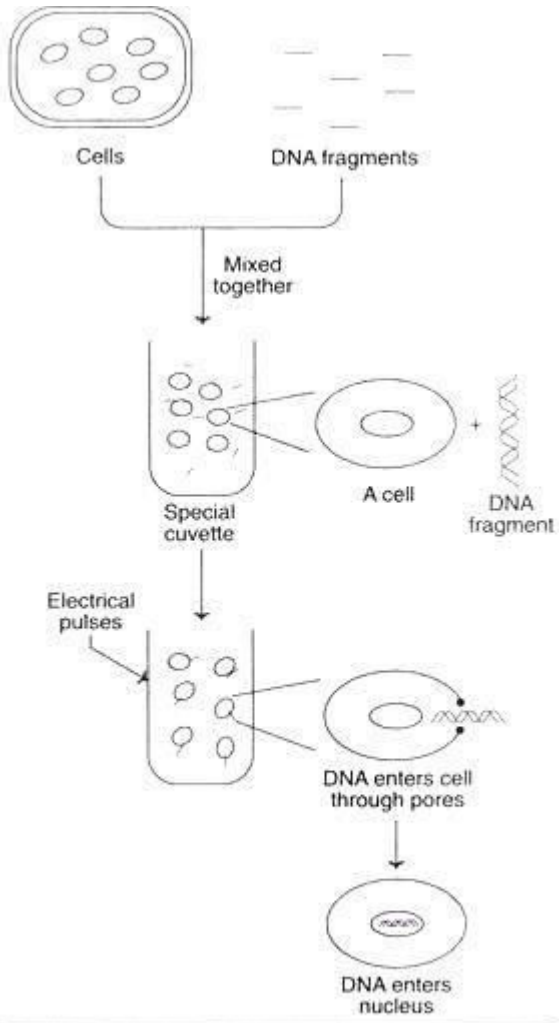


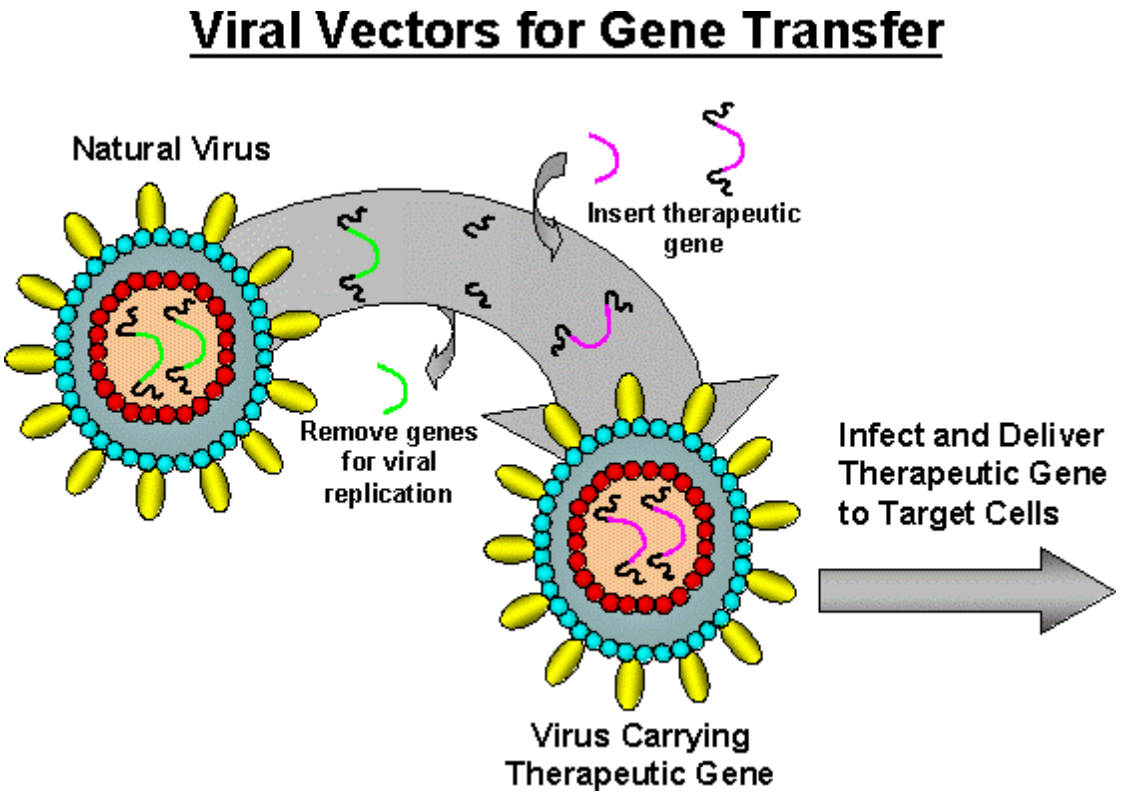
Fig. 6.11 : Gene transfer by electroporation.
(Note : Magnification depicted on right side)

VIRAL MEDIATED GENE TRANSFER TECHNIQUES

Viral vectors are tools commonly used by molecular biologists to deliver genetic material into cells. This process can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*). Viruses have evolved specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. Delivery of genes by a virus is termed transduction and the infected cells are described as transduced.

Key properties of a viral vector:

- *Safety*: Although viral vectors are occasionally created from pathogenic viruses, they are modified in such a way as to minimize the risk of handling them. This usually involves the deletion of a part of the viral genome critical for viral replication. Such a virus can efficiently infect cells but, once the infection has taken place, requires a helper virus to provide the missing proteins for production of new virions.
- *Low toxicity*: The viral vector should have a minimal effect on the physiology of the cell it infects.
- *Stability*: Some viruses are genetically unstable and can rapidly rearrange their genomes. This is detrimental to predictability and reproducibility of the work conducted using a viral vector and is avoided in their design.
- *Cell type specificity*: Most viral vectors are engineered to infect as wide a range of cell types as possible. However, sometimes the opposite is preferred. The viral receptor can be modified to target the virus to a specific kind of cell. Viruses modified in this manner are said to be pseudotyped.
- *Identification*: Viral vectors are often given certain genes that help identify which cells took up the viral genes. These genes are called Markers. A common marker is antibiotic resistance to a certain antibiotic. The cells can then be isolated easily as those that have not taken up the viral vector genes do not have antibiotic resistance and so cannot grow in a culture with antibiotics present.



TYPES OF VIRAL VECTORS:

RETROVIRUSES:

A retrovirus is a virus that carries its genetic material in the form of RNA rather than DNA. Retroviruses are used as vectors to transfer genetic material into the host cell. The result is a chimera, an organism consisting of tissues or parts of diverse genetic constitution. Chimeras are inbred for as many as 20 generations until homozygous genetic offspring are born.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

Recombinant retroviruses produce virions, which are used to infect animal cells and mice embryos. Generally, early 4-16 celled embryos are used. But when embryos beyond the 4-celled stage are used, often all the cells in an embryo may not be infected by the retrovirus, resulting in chimeric mice.

Retroviral vectors can either be replication-competent or replication-defective. Replication-defective vectors are the most common choice in studies because the viruses have had the coding regions for the genes necessary for additional rounds of virion replication and packaging replaced with other genes, or deleted. These virus are capable of infecting their target cells and delivering their viral payload, but then fail to continue the typical lytic pathway that leads to cell lysis and death.

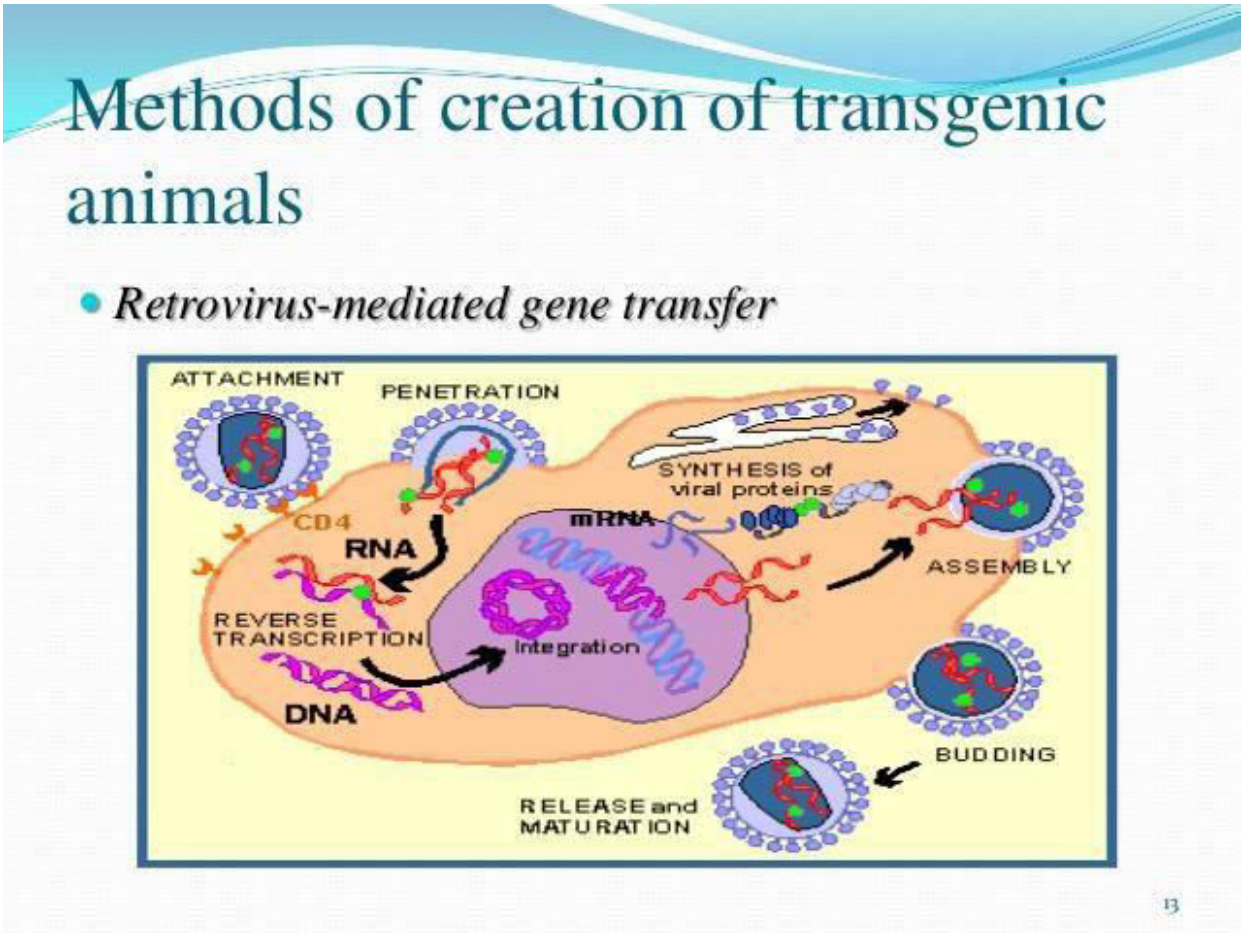
Conversely, replication-competent viral vectors contain all necessary genes for virion synthesis, and continue to propagate themselves once infection occurs. Because the viral genome for these vectors is much lengthier, the length of the actual inserted gene of interest is limited compared to the possible length of the insert for replication-defective vectors. Depending on the viral vector, the typical maximum length of an allowable DNA insert in a replication-defective viral vector is usually about 8–10 kB. While this limits the introduction of many genomic sequences, most cDNA sequences can still be accommodated.

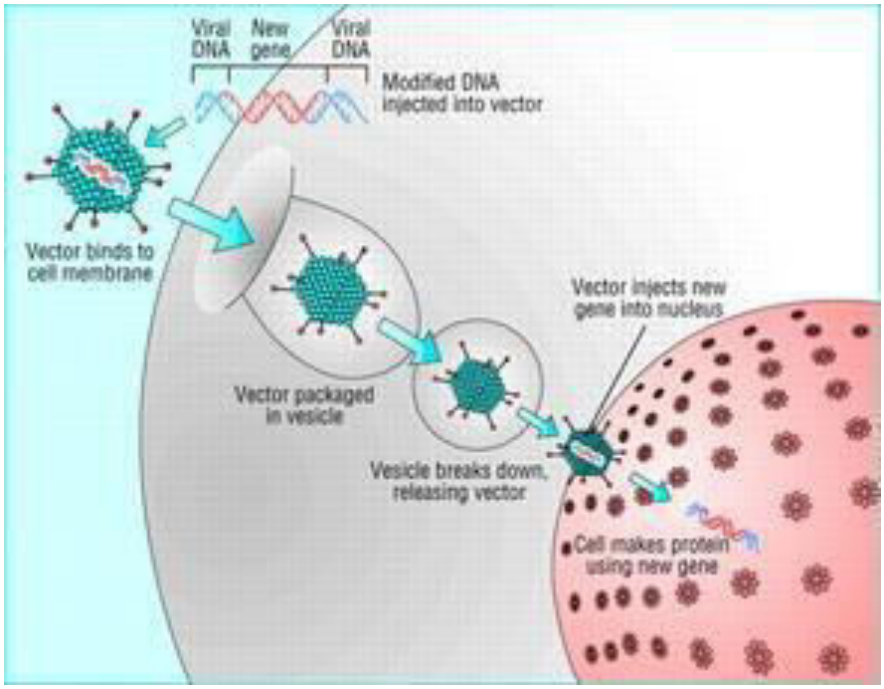
A chimera is an individual, which has in its body cells of two or more genotypes. Chimeric individuals produced by transfection arise when some cells of an embryo become stably transfected, while its other cells are not transfected; as a result, the individual developing from such an embryo will have cells of two genotypes in its body.

The recombinant retrovirus RNA genome is copied by reverse transcriptase to yield a DNA copy (reverse transcription), which becomes integrated into the cell's gemone. The reverse transcriptase is encoded by the retrovirus, and is produced immediately after infection.

However, the reverse transcription can occur only in those cells, which go through S phase, i.e., are mitotically active. The DNA copy of the recombinant retrovirus integrates into the cellular genome at random sites, and usually is not accompanied with deletions or rearrangements.

The chief advantage of this transfection method is its technical simplicity. Its main limitations, however, are as follows: (1) the additional steps needed for construction of recombinant retroviruses, (2) limits on the size of DNA insert, (3) masaicism of the recovered animals, and (4) possible interference of the proviral LTR sequences with the expression of foreign genes.



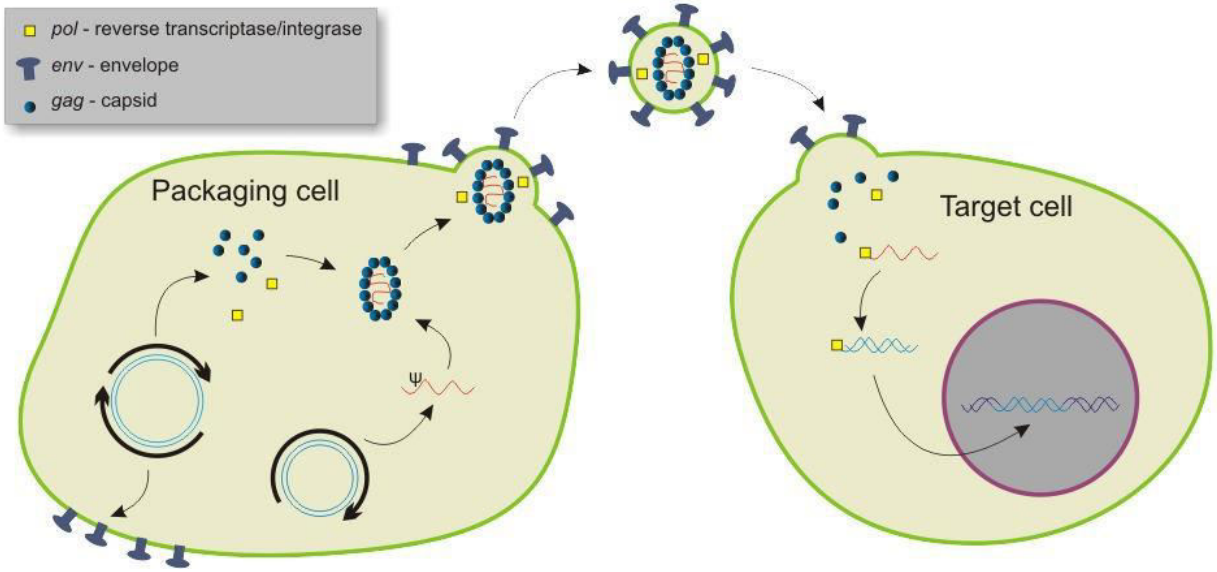


LENTIVIRUSES:

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 (recent findings actually suggest that the insertion of viral DNA is not random but directed to specific active genes and related to genome organisation) by the viral integrase enzyme. The vector, now called aprovirus, 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.[11] 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-calledpackaging 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



ADENOVIRUSES:

Adenoviral DNA does not integrate into the genome and is not replicated during cell division. This limits their use in basic research, although adenoviral vectors are still used in *in vitro* and also *in vivo* experiments. Their primary applications are in gene therapy and vaccination. Since humans commonly come in contact with adenoviruses, which cause respiratory, gastrointestinal and eye infections, majority of patients have already developed neutralizing antibodies which can inactivate the virus before it can reach the target

cell. To overcome this problem scientists are currently investigating adenoviruses that infect different species to which humans do not have immunity.

ADENO-ASSOCIATED VIRUSES:

Adeno-associated virus (AAV) is a small virus that infects humans and some other primate species. AAV is not currently known to cause disease and consequently the virus causes a very mild immune response. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Moreover, AAV mostly stays as episomal; performing long and stable expression. These features make AAV a very attractive candidate for creating viral vectors for gene therapy.[1] However, AAV can only bring up to 5kb which is considerably small compare to AAV's original capacity.

Furthermore, because of its potential use as a gene therapy vector, researchers have created an altered AAV called Self-complementary adeno-associated virus (scAAV). Whereas AAV packages a single strand of DNA and requires the process of second-strand synthesis, scAAV packages both strands which anneal together to form double stranded DNA. By skipping second strand synthesis scAAV allows for rapid expression in the cell. Otherwise, scAAV carries many characteristics of its AAV counterpart.

MOLECULAR PHARMING:

Pharming, a portmanteau of "farming" and "pharmaceutical", refers to the use of genetic engineering to insert genes that code for useful pharmaceuticals into host animals or plants that would otherwise not express those genes, thus creating a genetically modified organism (GMO).

Pharming is also known as molecular farming, molecular pharming or biopharming. The products of pharming are recombinant proteins or their metabolic products.

The production of pharmaceuticals in GM animals is another area of intensive research. The preferred bioreactor is the lactiferous gland. So far, more than 20 pharmaceuticals have been synthesised in the milk of mammals. Pharmaceutical agents can also be produced in chickens' eggs, blood, urine, and sperm. Since 2008 the first drug produced by transgenic animals is

availiable in the EU. Antithrombin is a substance that blocks blood clotting and can be extracted from the milk of genetically modified goats.

Pharming in mammals: Historical development:

Milk is presently the most mature system to produce recombinant proteins from transgenic organisms. Blood, egg white, seminal plasma, and urine are other theoretically possible systems, but all have drawbacks. Blood, cannot store high levels of stable recombinant proteins, and biologically active proteins in blood may alter the health of the animals. Expression in the milk of a mammal, such as a cow, sheep, or goat, is a common application, as milk production is plentiful and purification from milk is relatively easy. Hamsters and rabbits have also been used in preliminary studies because of their faster breeding.

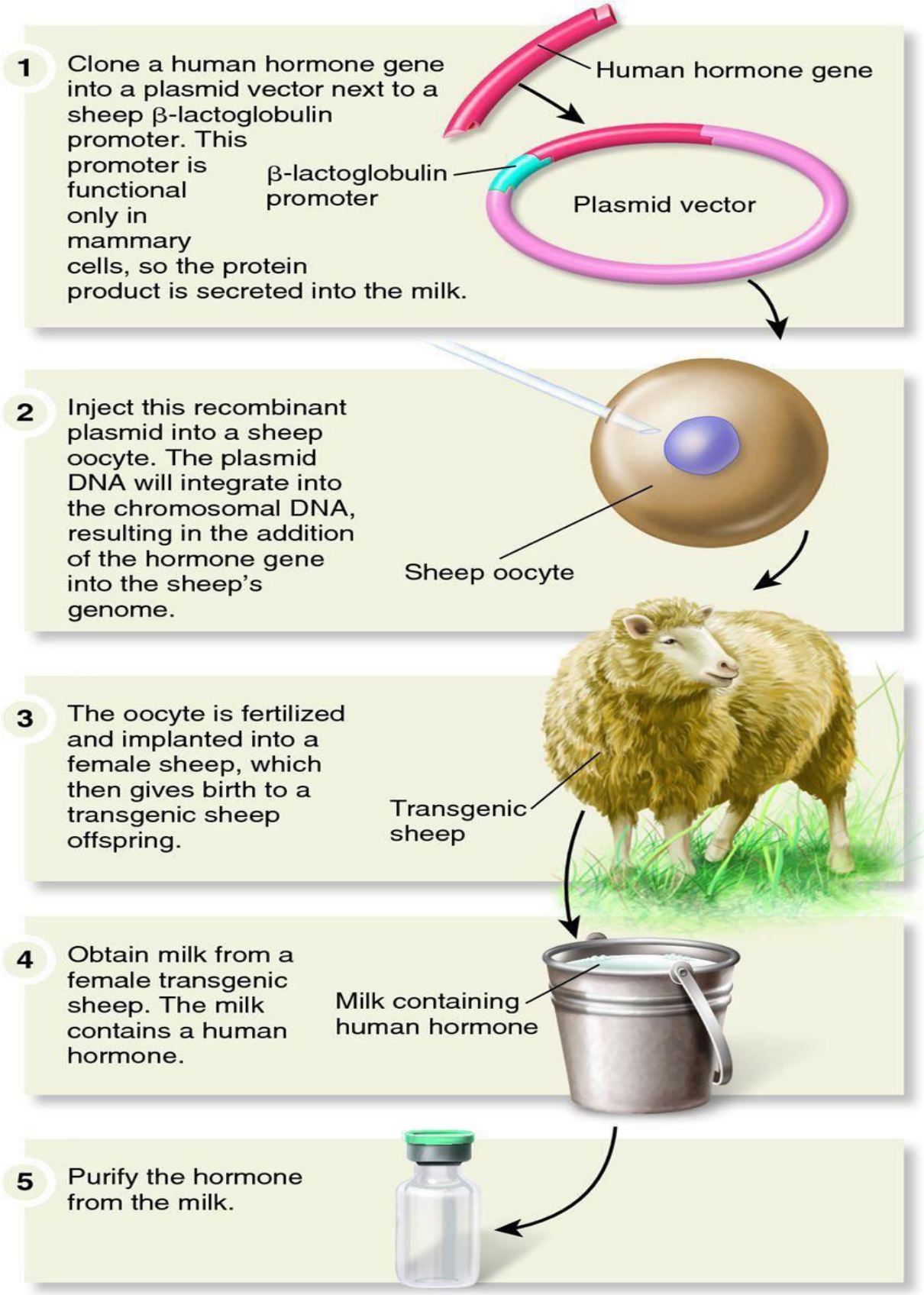
One approach to this technology is the creation of a transgenic mammal that can produce the biopharmaceutical in its milk (or blood or urine). Once an animal is produced, typically using the pronuclear microinjection method, it becomes efficacious to use cloning technology to create additional offspring that carry the favorable modified genome. The drug is called ATryn, which is antithrombin protein purified from the milk of genetically modified goats. Marketing permission was granted by the European Medicines Agency in August 2006.[20]

Patentability issues regarding pharming:

Some mammals typically used for food production (such as goats, sheep, pigs, and cows) have been modified to produce non-food products, a practice sometimes called pharming. Use of genetically modified goats has been approved by the FDA and EMA to produce ATryn, i.e. recombinant antithrombin, ananticoagulant protein drug. These products "produced by turning animals into drug-manufacturing 'machines' by genetically modifying them" are sometimes termed biopharmaceuticals.

The patentability of such biopharmaceuticals and their process of manufacture is uncertain. Probably, the biopharmaceuticals themselves so made are unpatentable, assuming that they are

chemically identical to the preexisting drugs that they imitate. Several 19th century United States Supreme Court decisions hold that a previously known natural product manufactured by artificial means cannot be patented.[22] An argument can be made for the patentability of the process for manufacturing a biopharmaceutical, however, because genetically modifying animals so that they will produce the drug is dissimilar to previous methods of manufacture; moreover, one Supreme Court decision seems to hold open that possibility.


1
Clone a human hormone gene into a plasmid vector next to a sheep β-lactoglobulin promoter. This promoter is functional only in mammary cells, so the protein product is secreted into the milk.
Human hormone gene
β-lactoglobulin promoter
Plasmid vector
2
Inject this recombinant plasmid into a sheep oocyte. The plasmid DNA will integrate into the chromosomal DNA, resulting in the addition of the hormone gene into the sheep's genome.
Sheep oocyte
3
The oocyte is fertilized and implanted into a female sheep, which then gives birth to a transgenic sheep offspring.
Transgenic sheep
4
Obtain milk from a female transgenic sheep. The milk contains a human hormone.
Milk containing human hormone
5
Purify the hormone from the milk.

GENE THERAPY

Gene therapy is the therapeutic delivery of nucleic acid polymers into a patient's cells as a drug to treat disease. Gene therapy is an experimental technique that uses genes to treat or prevent disease.

Gene therapy is when DNA is introduced into a patient to treat a genetic disease. The new DNAusually contains a functioning gene to correct the effects of a disease-causing mutation.The DNA is carefully selected to correct the effect of a mutated gene that is causing disease. Gene therapy may be a promising treatment option for somegenetic diseases[?], including muscular dystrophy[?] and cystic fibrosis[?].

- Replacing a mutated gene that causes disease with a healthy copy of the gene.
- Inactivating, or “knocking out,” a mutated gene that is functioning improperly.
- Introducing a new gene into the body to help fight a disease.
- Gene therapy is designed to introduce genetic material into cells to compensate for abnormal genes or to make a beneficial protein. If a mutated gene causes a necessary protein to be faulty or missing, gene therapy may be able to introduce a normal copy of the gene to restore the function of the protein.
- A gene that is inserted directly into a cell usually does not function. Instead, a carrier called a vector is genetically engineered to deliver the gene. Certain viruses are often used as vectors because they can deliver the new gene by infecting the cell. The viruses are modified so they can’t cause disease when used in people. Some types of virus, such as retroviruses, integrate their genetic material (including the new gene) into a chromosome in the human cell. Other viruses, such as adenoviruses, introduce their DNA into the nucleus of the cell, but the DNA is not integrated into a chromosome.
- The vector can be injected or given intravenously (by IV) directly into a specific tissue in the body, where it is taken up by individual cells. Alternately, a sample of the patient’s

cells can be removed and exposed to the vector in a laboratory setting. The cells

containing the vector are then returned to the patient. If the treatment is successful, the new gene delivered by the vector will make a functioning protein.

CELL TYPES: There are two different types of gene therapy depending on which types of cells are treated.

Gene therapy may be classified into two types:

Somatic cell:

In somatic cell gene therapy (SCGT), the therapeutic genes are transferred into any of any cell other than a gamete, germ cell, gametocyte or undifferentiated stem cell. Any such modifications affect the individual patient only, and are not inherited by offspring. Somatic gene therapy represents mainstream basic and clinical research, in which therapeutic DNA (either integrated in the genome or as an external episome or plasmid) is used to treat disease.

Germline:

In germline gene therapy (GGT), germ cells (sperm or eggs) are modified by the introduction of functional genes into their genomes. Modifying a germ cell causes all the organism's cells to contain the modified gene. The change is therefore heritable and passed on to later generations.

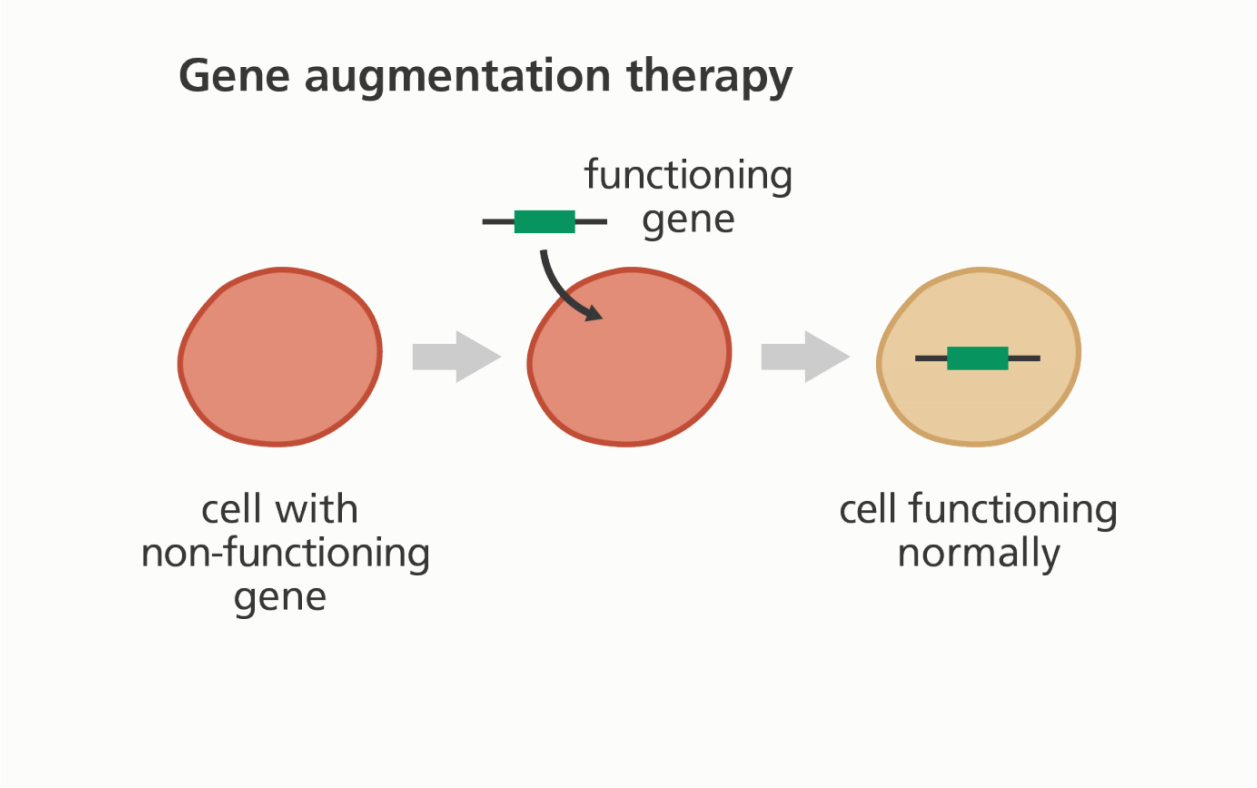
Gene Therapy Techniques- **There Are Several Techniques For Carrying Out Gene** Therapy. These Include:

Gene augmentation therapy:

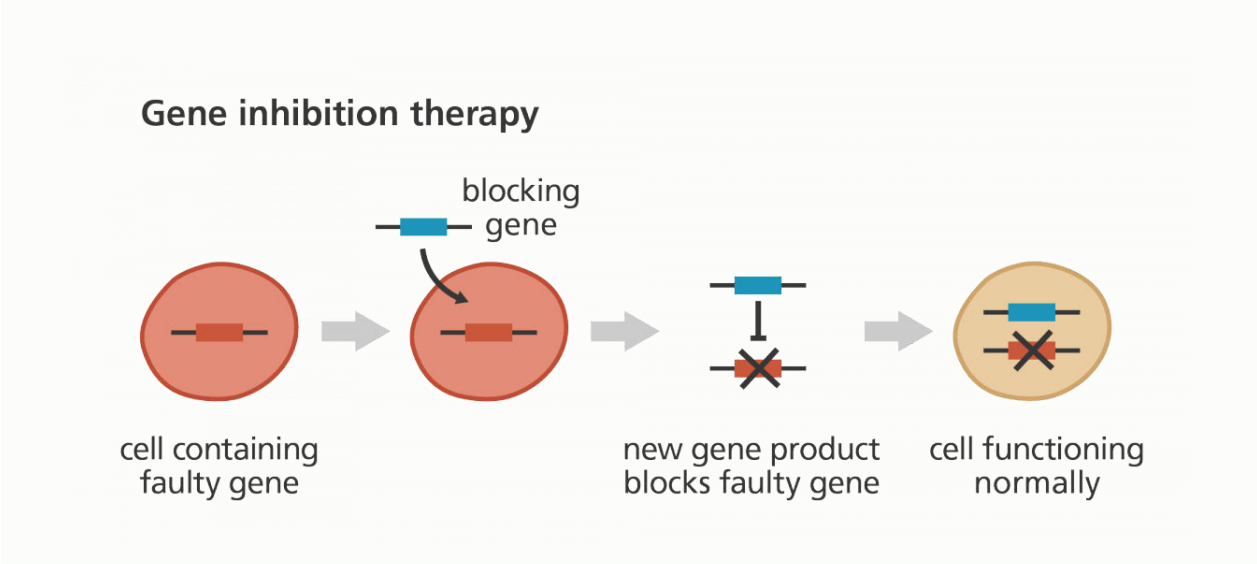
This is used to treat diseases caused by a mutation that stops a gene from producing a

- functioning product, such as a protein[?].
- This therapy adds DNA containing a functional version of the lost gene back into the cell.
- The new gene produces a functioning product at sufficient levels to replace the protein that was originally missing.
- This is only successful if the effects of the disease are reversible or have not resulted in lasting damage to the body.
- For example, this can be used to treat loss of function disorders such as cystic fibrosis by introducing a functional copy of the gene to correct the disease (see illustration

below).



- Suitable for the treatment of infectious diseases, cancer and inherited disease caused by inappropriate gene activity.
- The aim is to introduce a gene whose product either:
  - inhibits the expression of another gene
  - interferes with the activity of the product of another gene.
- The basis of this therapy is to eliminate the activity of a gene that encourages the growth of disease-related cells.
- For example, cancer is sometimes the result of the over-activation of an oncogene? (gene which stimulates cell growth). So, by eliminating the activity of that oncogene through gene inhibition therapy, it is possible to prevent further cell growth and stop



The aim is to insert DNA into a diseased cell that causes that cell to die.

Suitable for diseases such as cancer that can be treated by destroying certain groups of cells.

This can be achieved in one of two ways:

the inserted DNA contains a "suicide" gene that produces a highly toxic product which kills the diseased cell

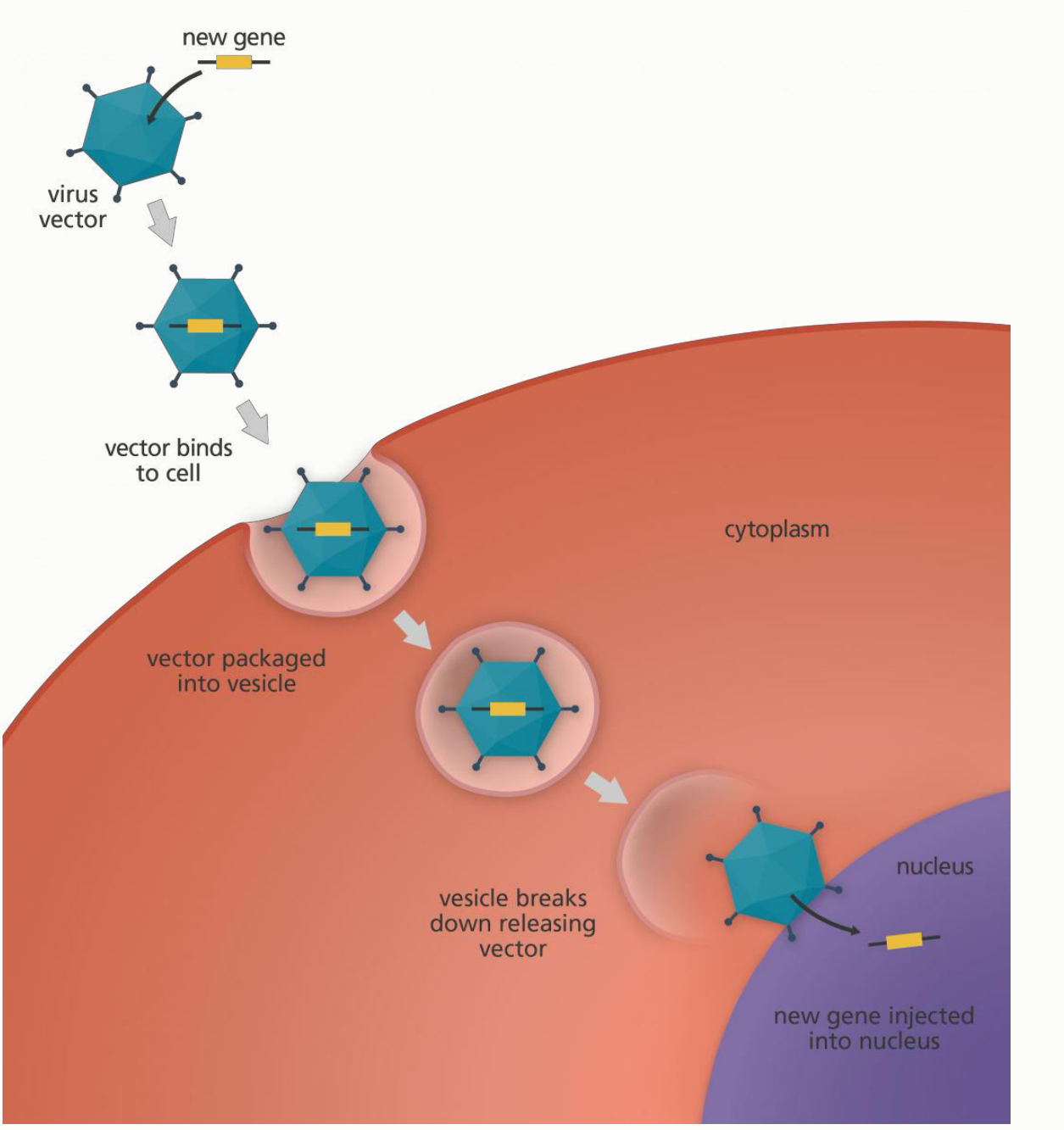
the inserted DNA causes expression of a protein that marks the cells so that the diseased cells are attacked by the body's natural immune system.

It is essential with this method that the inserted DNA is targeted appropriately to avoid the death of cells that are functioning normally

How is DNA transfer done?

- A section of DNA/gene containing instructions for making a useful protein is packaged within a vector, usually a virus[?],bacterium[?] or plasmid[?].
- The vector acts as a vehicle to carry the new DNA into the cells of a patient with a genetic disease.

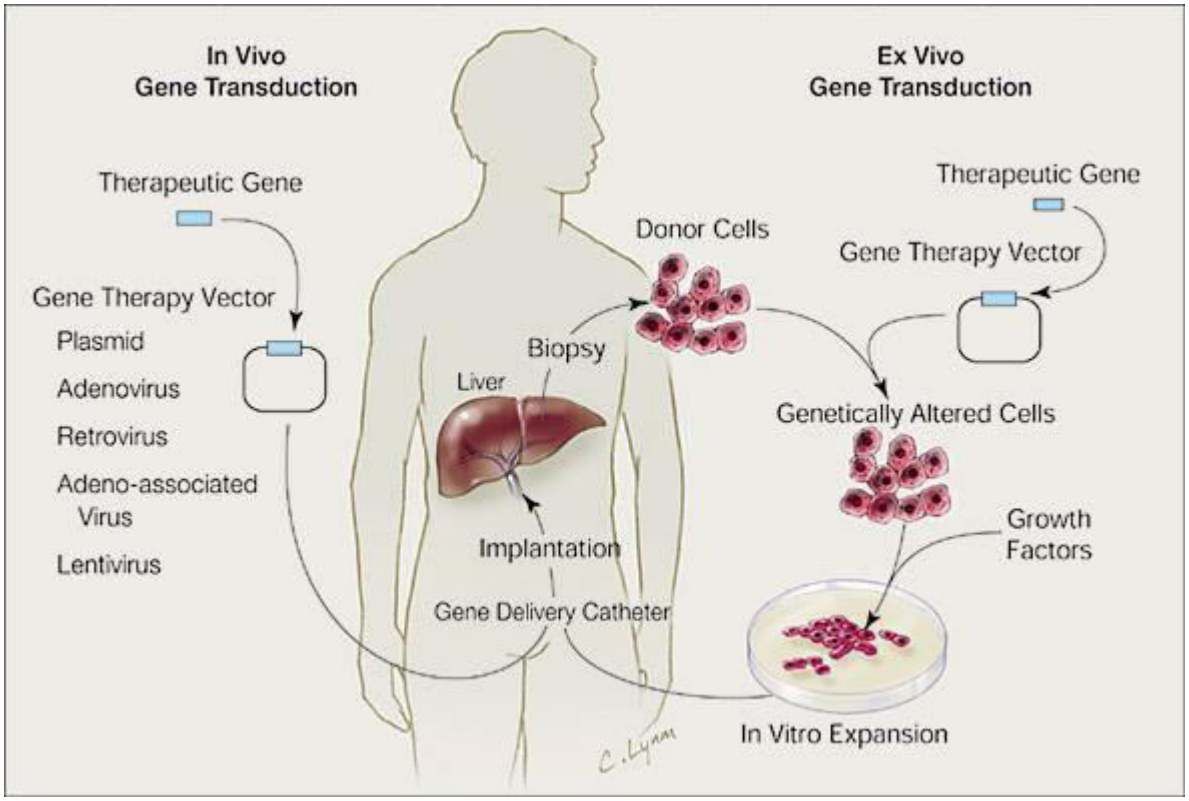
- Once inside the cells of the patient, the DNA/gene is expressed by the cell's normal machinery leading to production of the therapeutic protein and treatment of the patient's disease.



Somatic gene therapy can be broadly split into two categories:

Ex Vivo- which means exterior (where cells are modified outside the body and then transplanted back in again). In some gene therapy clinical trials, cells from the patient's blood or bone marrow are removed and grown in the laboratory. The cells are exposed to the virus that is carrying the desired gene. The virus enters the cells and inserts the desired gene into the cells' DNA. The cells grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene therapy is called ex vivo because the cells are treated outside the body.

In Vivo, which means interior (where genes are changed in cells still in the body). This form of gene therapy is called in vivo, because the gene is transferred to cells inside the patient's body.

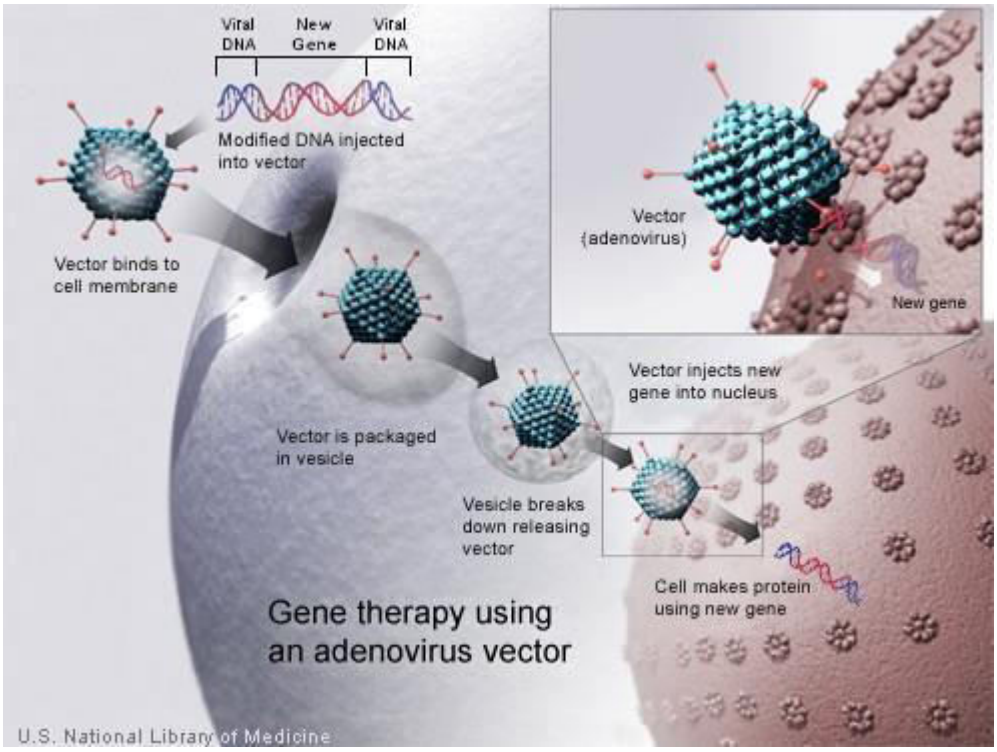


VECTORS:

The delivery of DNA into cells can be accomplished by multiple methods. The two major classes are recombinant viruses (sometimes called biological nanoparticles or viral vectors) and naked DNA or DNA complexes (non-viral methods).

Viruses

In order to replicate, viruses introduce their genetic material into the host cell, tricking the host's cellular machinery into using it as blueprints for viral proteins. Scientists exploit this by substituting a virus's genetic material with therapeutic DNA. (The term 'DNA' may be an oversimplification, as some viruses contain RNA, and gene therapy could take this form as well.) A number of viruses have been used for human gene therapy, including retrovirus, adenovirus, lentivirus, herpes simplex,vaccinia and adeno-associated virus.[3] Like the genetic material (DNA or RNA) in viruses, therapeutic DNA can be designed to simply serve as a temporary blueprint that is degraded naturally or (at least theoretically) to enter the host's genome, becoming a permanent part of the host's DNA in infected cells.



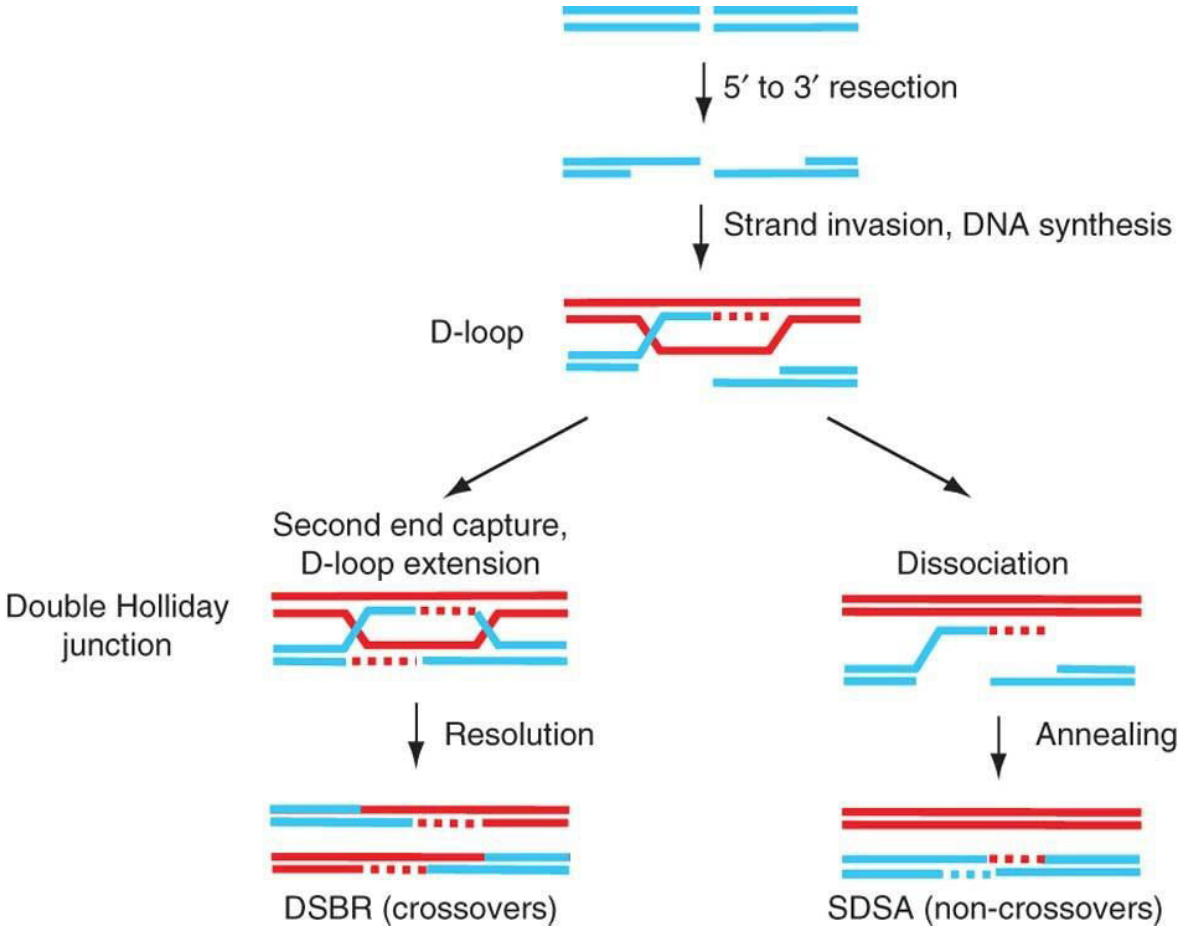
Non-viral:

Non-viral methods present certain advantages over viral methods, such as large scale production and low host immunogenicity. However, non-viral methods initially produced lower levels of transfection and gene expression, and thus lower therapeutic efficacy. Later technology remedied this deficiency.

Methods for non-viral gene therapy include the injection of naked DNA, electroporation, the gene gun, sonoporation, magnetofection, the use of oligonucleotides, lipoplexes, dendrimers, and inorganic nanoparticles.

GENE TARGETING

Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA, known as double-strand breaks. Homologous recombination also produces new combinations of DNA sequences during meiosis, the process by which eukaryotes make gamete cells, like spermand egg cells in animals. These new combinations of DNA represent genetic variation in offspring, which in turn enables populations to adapt during the course of evolution.[1] Homologous recombination is also used in horizontal gene transfer to exchange genetic material between different strains and species of bacteria and viruses.


5′ to 3′ resection
Strand invasion, DNA synthesis
D-loop
Second end capture,
D-loop extension
Dissociation
Double Holliday
junction
Resolution
Annealing
DSBR (crossovers)
SDSA (non-crossovers)

Gene targeting (also, replacement strategy based on homologous recombination) is a genetic technique that uses homologous recombination to change an endogenous gene. The method can be used to delete a gene, remove exons, add a gene, and introduce point mutations. Gene targeting can be permanent or conditional. Conditions can be a specific time during development / life of the organism or limitation to a specific tissue, for example. Gene targeting requires the creation of a specific vector for each gene of interest. However, it can be used for any gene, regardless of transcriptional activity or gene size.

Transgenic Mouse: Generic term for an engineered mouse that has a normal DNA sequence for a gene replaced by an engineered sequence or a sequence from another organism.

Knockout Mouse: A transgenic mouse in which the normal gene is missing or engineered so that is not transcribed or translated. "Knocks out" that gene.

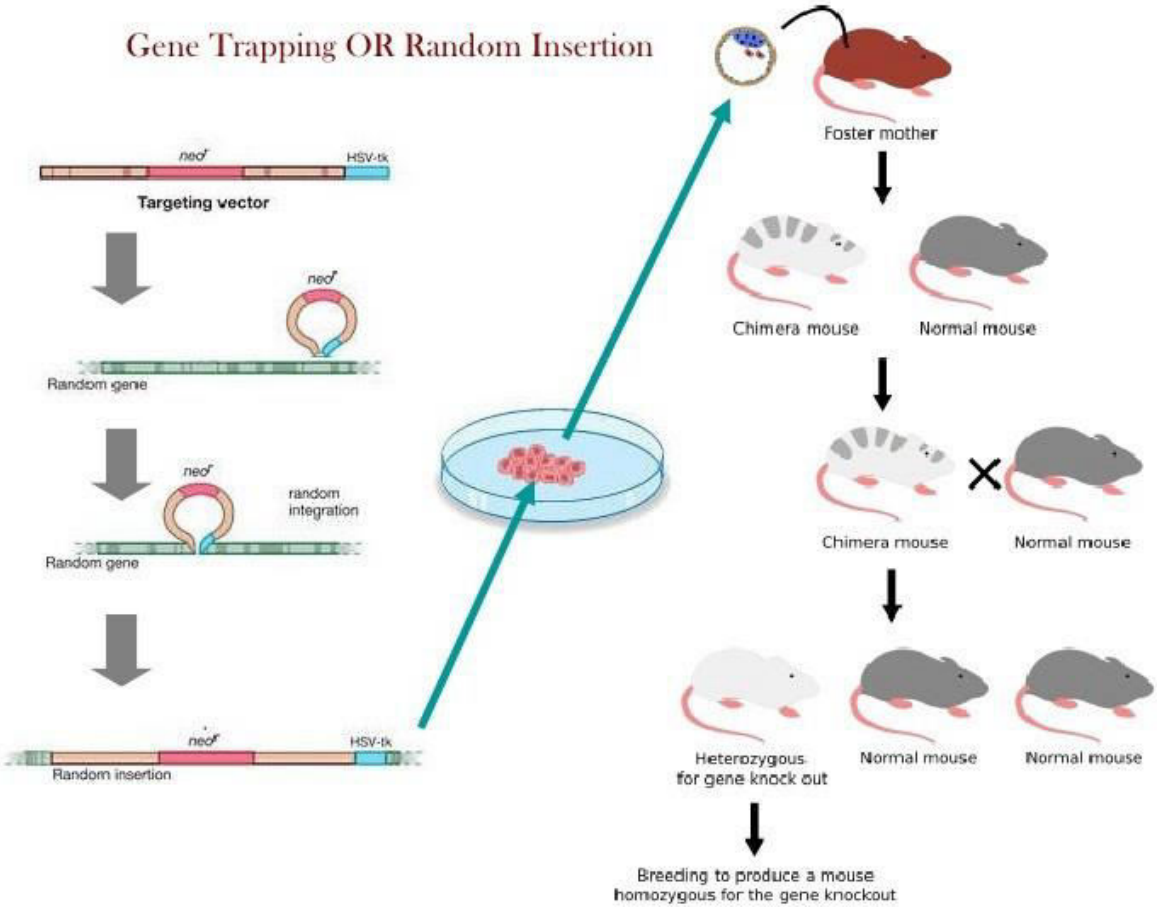
Knockin Mouse: A transgenic mouse in which the engineered "transgene" is subtly manipulated to: (A) alter the function of the gene (e.g., replace one amino acid with another in a site to determine if that site is essential for the protein's function); (B) change transcription rate to overproduce or underproduce the gene product; or (C) create a fluorescent gene product to map its distribution in tissue.

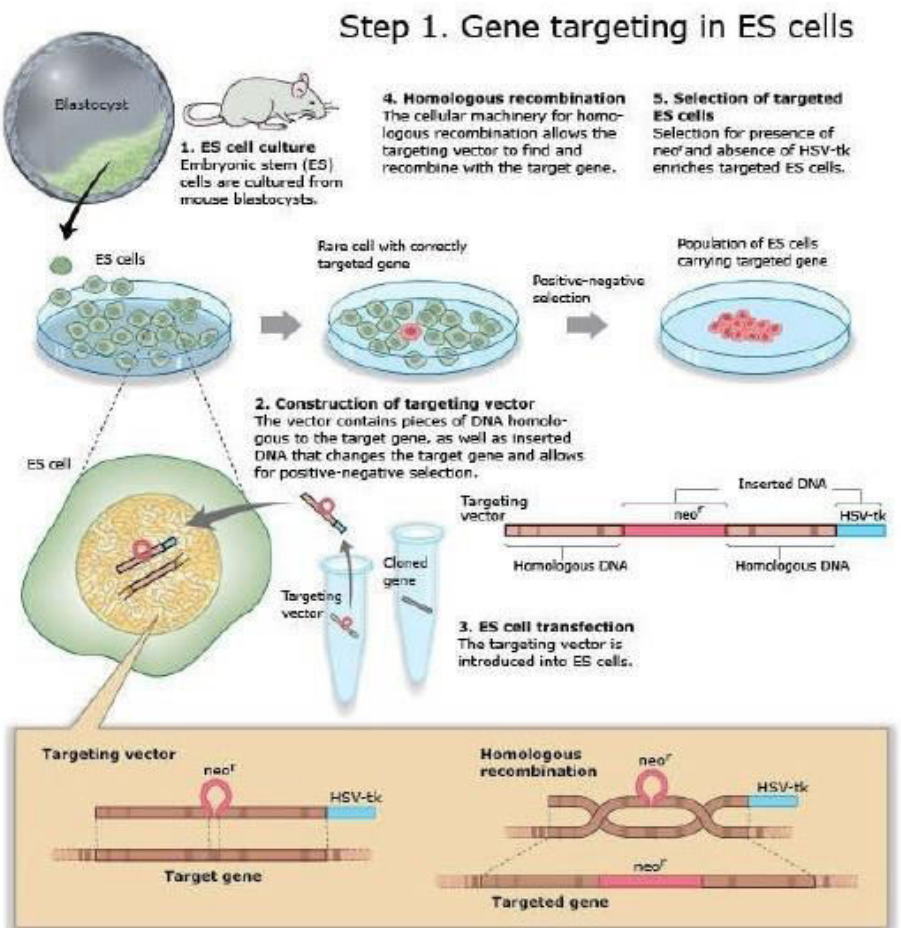
Conditional Knockout (Knockin) Mouse: A transgenic mouse in which the transgene is knocked out (or in) in specific tissues, at a specific developmental stage, or in response to an exogenous substance (e.g., an antibiotic).

The First Knockout mouse was created by Mario R Capecchi,Martin Evans and Oliver Smithies in 1989 for which they were awarded Nobel Prize for Medicine in 2007.

RANDOM GENE INSERTION

The classic method used for the generation of transgenic mice is called « pronuclear injection ». The transgene is injected into a fertilized mouse egg and then integrates at random positions in the genome.





TARGETED GENE INSERTION

The early vectors used for gene insertion could, and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that

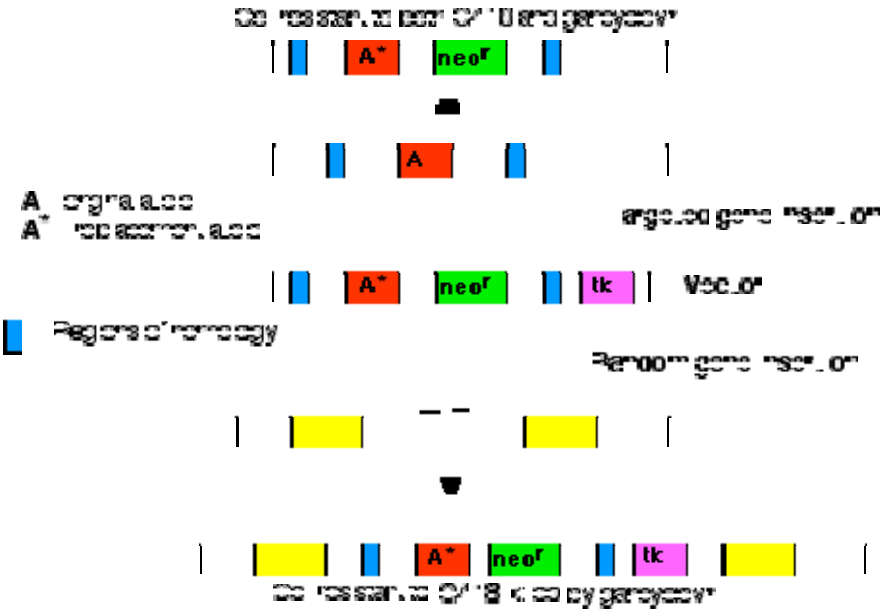
- restores function in a mutant animal or
- knocks out the function of a particular locus.

In either case, targeted gene insertion requires

- the desired gene

- ***neo***^{r}, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells;

- ***tk***, a gene that encodes **thymidine kinase**, an enzyme that phosphorylates the nucleoside analog **ganciclovir**. **DNA polymerase** fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So ganciclovir kills cells that contain the *tk* gene.



Step 1: Treat culture of ES cells with preparation of vector DNA.

Results:

- **Most cells** fail to take up the vector; these cells will be killed if exposed to G418.

- In a **few cells**: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the ***tk*** gene, is inserted into host DNA. These cells are resistant to G418 but killed by gancyclovir.

- In **still fewer cells**: homologous recombination occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome, and the region between these homologous sequences replaces the equivalent region in the host DNA.

Step 2 : Culture the mixture of cells in medium containing both G418 and ganciclovir.

- The cells (the majority) that failed to take up the vector are killed by G418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the *tk* gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).
- **Step 3:**Inject these into the inner cell mass of mouse blastocysts.

Knockout Mice:

Step 1: Generating a targeting vector

The first step in making a knockout mouse is identifying the region of the gene that will be deleted. Because the entire mouse genome sequence is already known, it is relatively simple to look up the chromosomal location and nucleic acid sequence of the gene of interest. Once the segment of the gene that will be deleted has been mapped out, the nucleic acid sequences of the DNA segments that appear on the chromosome before and after that gene must also be identified.

Once these tasks are completed, a targeting vector specifically tailored to the gene of interest is made (Figure 1). A targeting vector is a long stretch of DNA made up of smaller pieces of DNA that have been joined together. Next, in order to make the targeting vector detectable, the scientists insert a marker gene into the middle of the vector; this marker is in some way able to "report" when it is present in a cell.

Currently, the neomycin-resistance gene, called *NeoR*, is a popular marker gene of choice for generating knockout mice. The antibiotic neomycin is toxic to mouse cells because they do not normally contain the *NeoR* gene. However, when the *NeoR* gene is added to mouse cells, these cells can survive in the presence of neomycin. Within the targeting vector, the *NeoR*gene is located between two other pieces of DNA: the "right arm" and the "left arm" of the targeting vector. The right arm of the targeting vector contains DNA with a nucleic acid sequence that matches the stretch of DNA immediately *before* the gene segment that will be deleted. The left arm of the targeting vector contains DNA with a nucleic acid sequence that matches the stretch of DNA immediately *after* the gene segment that will be deleted. The right and left arms of the

targeting vector facilitate homologous recombination between the targeting vector and the target gene, thereby enabling the *NeoR* gene to replace the target gene segment.

The targeting vector also contains one additional piece of DNA, called a **negative selection marker gene**. This gene is located at the right end of the targeting vector, after the right arm. The thymidine kinase (*TK*) gene from the herpes simplex virus is the most commonly used negative selection marker gene. Normally, mouse cells can grow in the presence of the antiviral drug ganciclovir. The *TK* gene is considered a "cell suicide gene," however, as cells containing the *TK* gene convert ganciclovir into a lethal toxin.

Why is it necessary to include a cell suicide gene as part of the targeting vector? The reason is purely a matter of identification - specifically, the *TK* gene helps researchers locate cells that have correctly replaced the targeted gene segment with the *NeoR* gene. Often, mouse cells randomly insert the targeting vector in the wrong chromosomal location. If random insertion occurs, both the *NeoR* gene and the *TK* gene are inserted into the genome. As a result, the cells are resistant to neomycin, but they die in the presence of ganciclovir. In comparison, when the targeted gene segment is correctly replaced, the *TK* gene is not inserted into the chromosome along with the *NeoR* gene, so the resultant cells are resistant to both neomycin and ganciclovir. Therefore, the presence of the *TK* gene in the targeting vector allows researchers to efficiently screen for mouse cells that have correctly replaced the targeted gene segment by growing these cells in the presence of both neomycin and ganciclovir.

Step 2: Inserting the target sequence and selecting cells with the insertion

After the targeting vector is made, it is used to knock out one copy of the target gene in mouse embryonic stem (ES) cells

It is certainly possible to use the targeting vector to knock out one of the two copies of the target gene in a standard somatic cell. However, unless that cell is an ES cell, the knockout mutation cannot be incorporated into a growing embryo. Therefore, it would not be possible to study the effects of the knockout mutation in a developing mouse.

What makes ES cells so special? Primarily, it is their ability to become any one of the different adult cell types. When injected into a mouse embryo, the ES cells themselves are capable of maturing into some of the tissues of the developing mouse.A technique called **electroporation** is used. When ES cells are electroporated, a brief pulse of an electrical field is applied to the outside of the cells, creating a momentary increase in plasma membrane permeability and allowing the uptake of foreign DNA into the ES cells.

After the ES cells have been electroporated, they are grown in the presence of neomycin to select for those particular cells that have taken up the targeting vector. Next, the neomycin-resistant cells are grown in the presence of ganciclovir to select for those that have inserted the targeting vector at the correct location within the mouse genome.

Step 3: Identifying ES cells with the correct gene knocked out

Additional experiments using standard molecular biology techniques help researchers determine whether the target gene has been fully knocked out in the ES cells that are resistant to both neomycin and ganciclovir. After these experiments are complete, only the ES cells that have had one copy of the target gene knocked out remain .These ES cells are heterozygous for the knockout mutation. Although these cells will grow and divide in culture, they cannot form an embryo that will develop into a mouse on their own.

Step 4: Injecting heterozygous knockout ES cells into a developing embryo and transferring the embryo into a mouse

The first step involves injection of these cells into a developing mouse embryo. This step allows the heterozygous knockout ES cells to become part of the developing embryo. Then, because they are ES cells, the heterozygous knockout cells are incorporated throughout the embryo and are capable of becoming any type of tissue within the developing mouse. Therefore, like a patchwork quilt, the developing embryo contains a mixture of its own original cells and the heterozygous knockout cells. Because of this cellular mixing, the resultant mouse is called a **chimeric mouse.**

Step 5: Mating chimeric mice to yield homozygous knockout mice

To produce a mouse that is homozygous for the target gene knockout, chimeric mice capable of passing the knockout mutation on to their offspring must be identified.

These chimeric mice can be identified by crossing them with normal white mice. If black offspring are produced from such a cross, a chimeric mouse is capable of passing the knockout mutation on to its offspring. When this is the case, 50% of the black offspring are heterozygous for the knockout mutation in all of their cells

Standard molecular biology techniques can be used to determine which of the black offspring are heterozygous for the knockout mutation. Then, to produce a homozygous knockout mouse, a heterozygous knockout male is mated to a heterozygous knockout female.Twenty-five percent of the resulting offspring will be homozygous knockout mice, which can, again, be readily identified using standard molecular biology techniques.

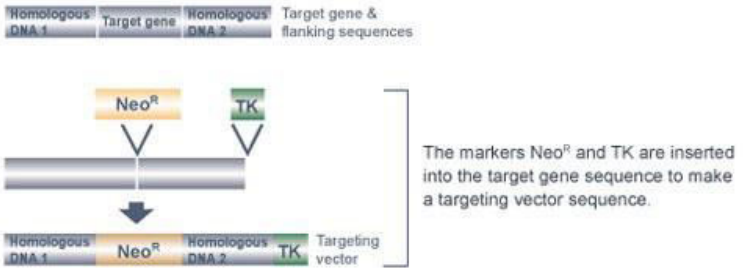
Step 6: Phenotypic characterization of homozygous knockout mice

After the homozygous knockout mice have been created, researches must then characterize the phenotypes associated with the loss of the target gene. In theory, every measurable phenotype must be examined in order to determine every possible function of the knocked-out gene. The measurement of a given phenotype in a knockout mouse would then be compared to measurements of the same phenotype in a **wild-type mouse** (a mouse that has not been genetically engineered for specific traits) in order to identify the functions that are altered in the knockout mouse.

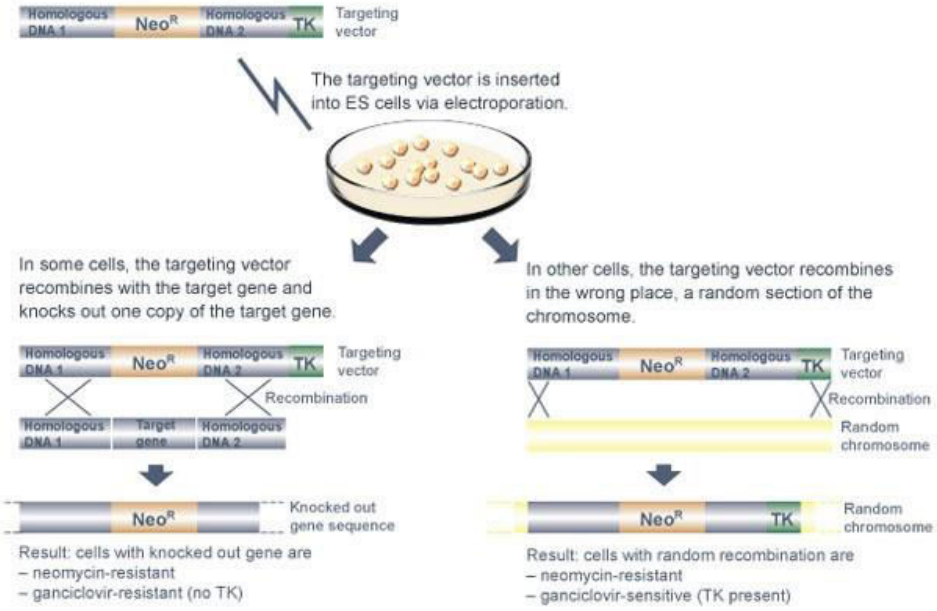
Phenotypes such as size, weight, metabolism, behavior, bone development, neurological function, reproduction, and aging can be easily measured. If the knocked-out gene is required for development, however, it may not be possible to produce homozygous knockout mice. In this case, researchers may study heterozygous knockout mice, or they may instead turn to other types of knockout mice, such as **conditional knockout mice** (in which the target gene is inactivated in response to a specific stimulus) or **tissue-specific knockout mice** (in which the target gene is inactivated in only one or several tissues).

Making a knockout mouse

Step 1: Designing the targeting vector



Step 2: Inserting the targeting vector into ES cells



Step 3: Selecting cells

Only the cells that have successfully incorporated the targeting vector into the target gene survive in the presence of neomycin and ganciclovir (shown in red).

Step 4: Injecting cells into a new embryo

Cells containing the targeting vector are then selected and injected into a normal developing mouse embryo.

Step 5: Breeding

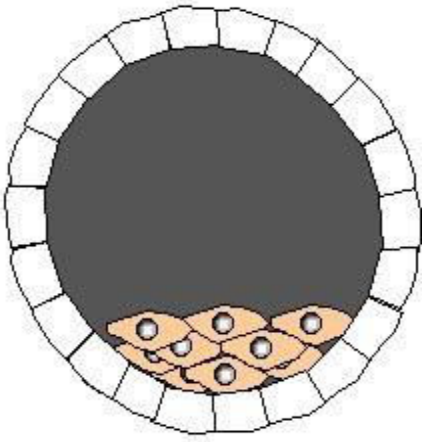
The resulting chimeric (spotted) mouse contains a mix of its own cells and the heterozygous knockout cells. This mouse is bred with a normal (white) mouse.

Among their offspring are mice that are capable of passing the knocked-out gene

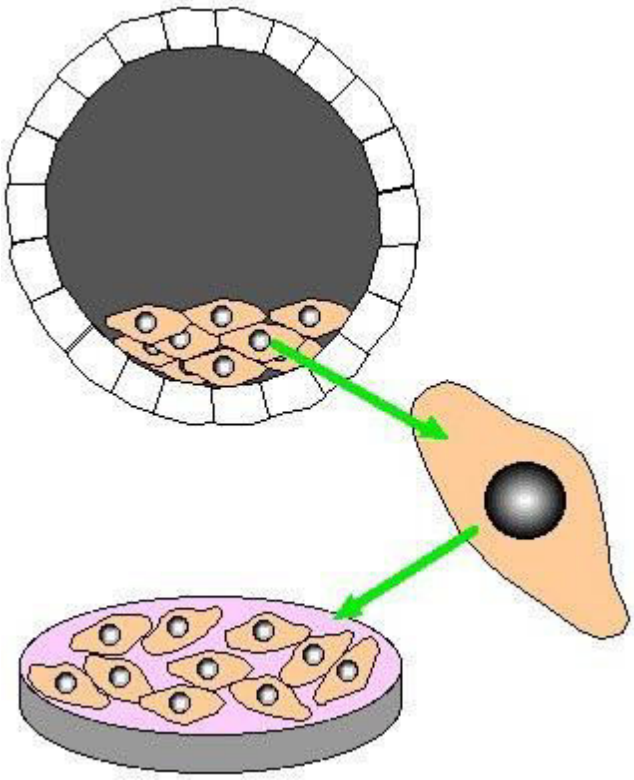
KNOCKOUT MOUSE

A **knockout mouse** has had both alleles of a particular gene replaced with an inactive allele. This is usually accomplished by using homologous recombination to replace one allele followed by two or more generations of selective breeding until an breeing pair are isolated that have both alleles of the targeted gene inactivaated or knocked out. Knock out mice allow investigators determine the role of a particular gene by observing the phenotype of individuals that lack the gene completely.

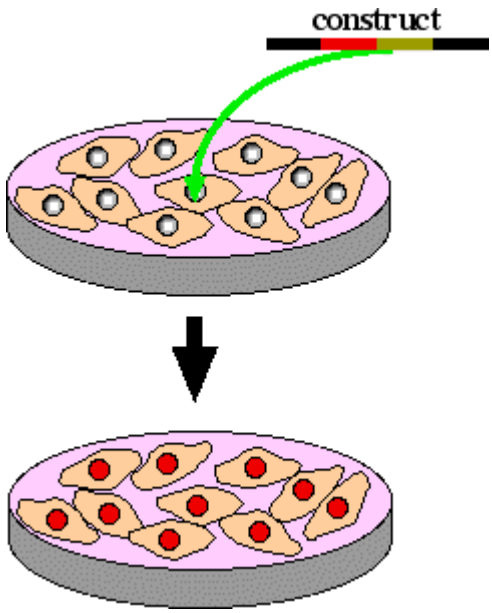
Step 1. Isolate developing embryo at blastocyst stage. This embryo is from a strain of mice with gray fur.



Step 2. Remove embryonic stem cells from gray-fur blastocyst. Grow stem cells in tissue culture.



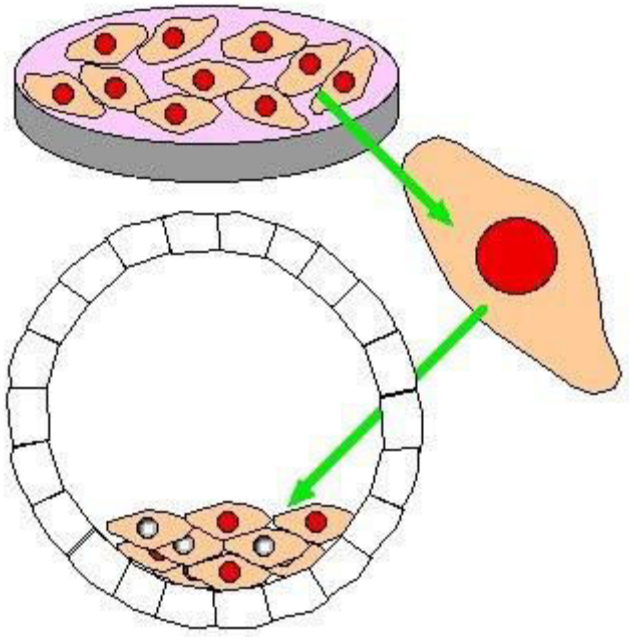
Step 3. Transfect stem cells with homologous recombination construct. Select for homologous recombination by growing stem cells in neomycin and gancyclvir.



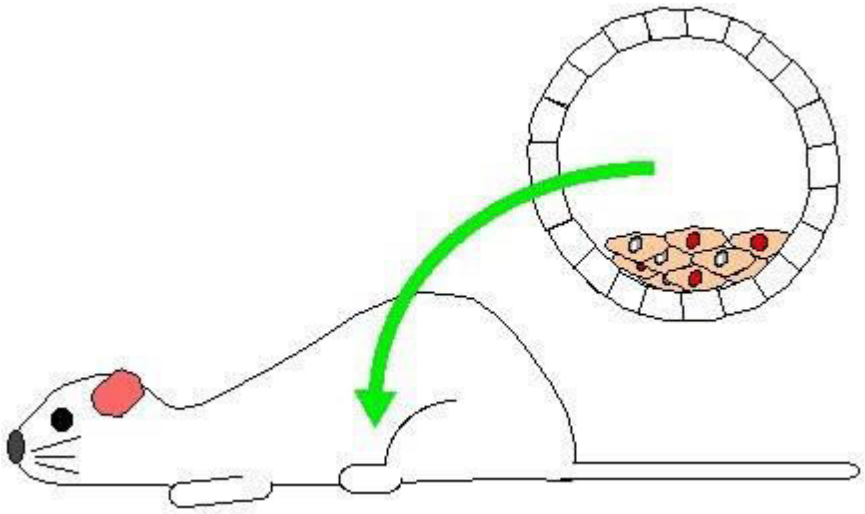
+ +

neomycin gancyclovir

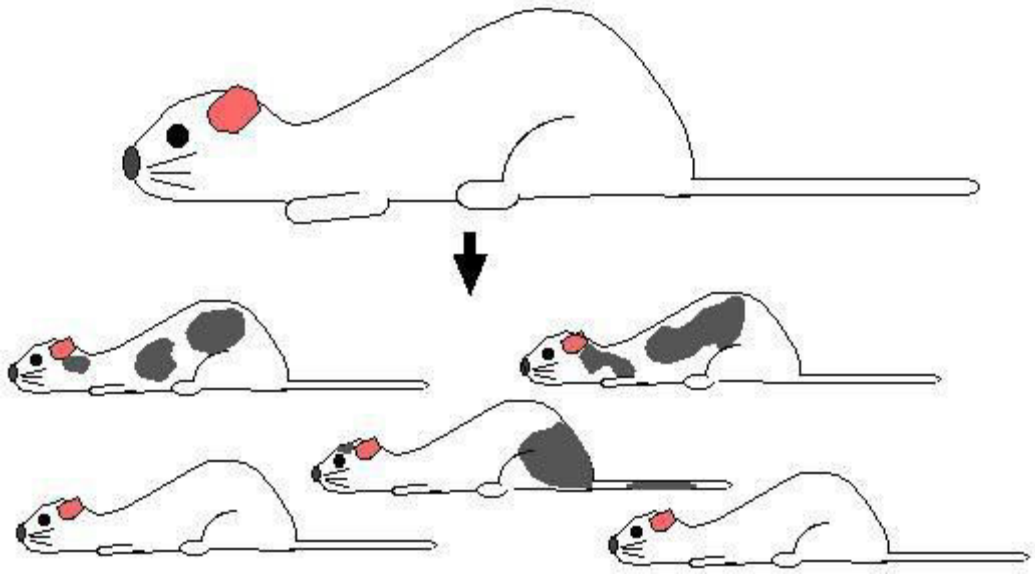
Step 4. Remove homologously recombined stem cells from petri dish and inject into a new blastocyst that would have only white fur.



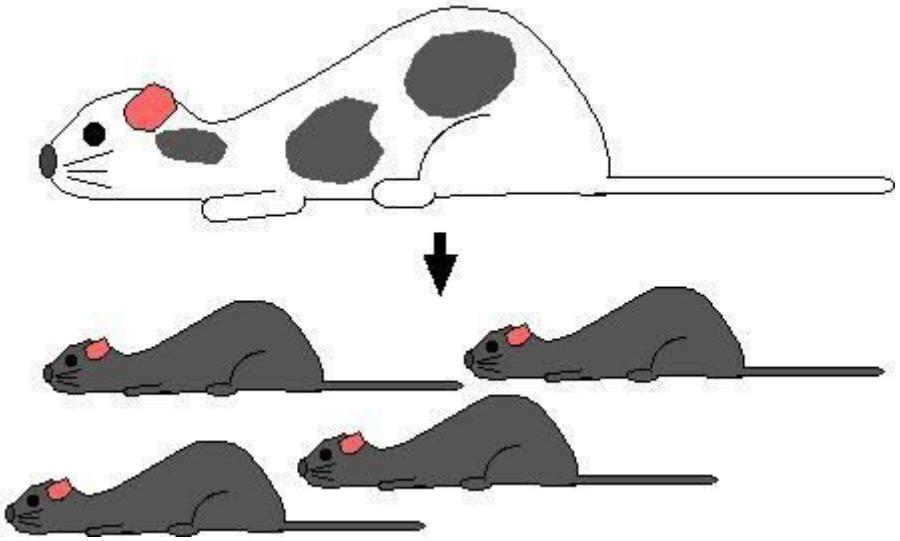
Step 5. Implant several chimeric blastocysts into pseudo-pregnant, white fur mouse.



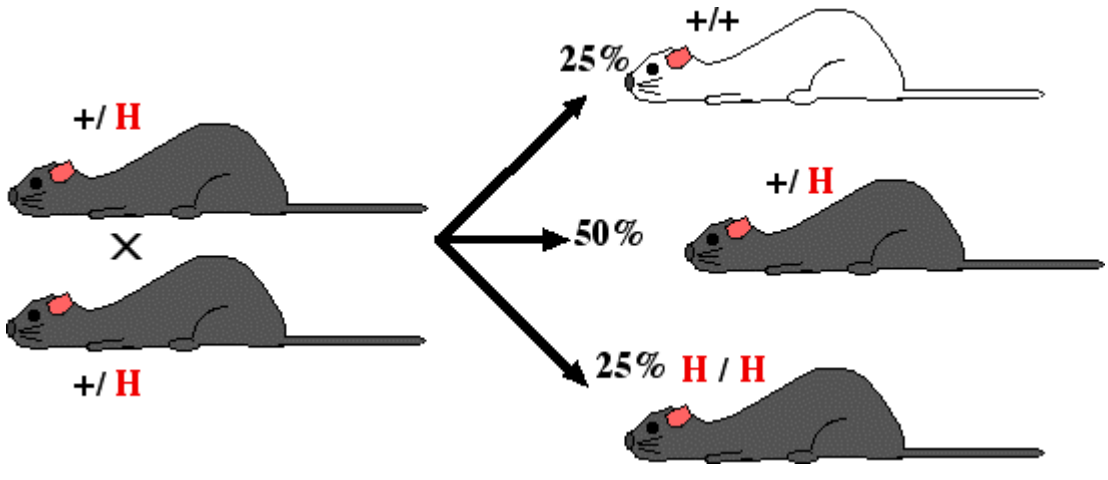
Step 6. Mother will give birth to a range of mice. Some will be normal white fur mice but others will be chimeric mice. Chimeric mice have many of their cells from the original white fur blastocyst but some of their cells will be derived from recombinant stem cells. Fur cells from recombinant stem cells produce gray patches which are easily detected.



Step 7. Mate the chimeric mice with wild-type white fur mice. If the gonads of the chimeric mice were derived from recombinant stem cells, all the offspring will have gray fur. Every cell in gray mice are heterozygous for the homologous recombination.



Step 8. Mate heterozygous gray mice (+/ H) and genotpye the gray offspring. Identify homozygous recombinants (H / H) and breed them to produce a strain of mice with both alleles knocked out. The pure breeding mouse strain is a "knockout mouse".



knockout mouse

KNOCK-IN MICE

The Cre/*loxP* system can also be used to

- remove DNA sequences that block gene transcription. The "target" gene can then be turned **on** in certain cells or at certain times as the experimenter wishes.
- replace one of the mouse's own genes with a new gene that the investigator wishes to study.

Such transgenic mice are called "knock-in" mice.

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SCHOOL OF BIO AND CHEMICAL ENGINEEING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – ANIMAL BIOTECHNOLOGY – SBT1305

UNIT IV IN VITRO FERTILIZATION TECHNIQUES

Manipulation of reproduction in animals-artificial insemination, embryo transfer. invitro fertilization technology- invitro maturation of oocytes, culture of invitro fertilized embryos, embryo cloning- quadriparental hybrid, nuclear transplantation (Dolly), embryonic stem cells.

ARTIFICIAL INSEMINATION:

It is the deliberate introduction of sperm into a female's uterus for the purpose of achieving a pregnancy through in vivo fertilization. It is a fertility treatment for humans, as well as in animal breeding, including dairy cattle and pigs.

Artificial insemination may employ assisted reproductive technology. Artificial insemination techniques available include intracervical insemination and intrauterine insemination. Assisted reproductive technology (ART) is the technology used to achieve pregnancy in procedures such as fertility medication, artificial insemination, in vitro fertilization and surrogacy. It is reproductive technology used primarily for infertility treatments.

Assisted reproductive technology (**ART**) is the **technology** used to achieve pregnancy in procedures such as fertility medication, artificial insemination, in vitro fertilization and surrogacy. It is **reproductive technology** used primarily for infertility treatments, and is also known as fertility treatment.

PROCEDURES: General

With ART, the process of sexual intercourse is bypassed either by artificial insemination or fertilization of the oocytes in the laboratory environment (i.e., in vitro fertilization). In general, ART procedures involve surgically removing eggs from a woman's ovaries, combining them with sperm in the laboratory, and returning them to the woman's body or donating them to another woman." According to CDC, "they do not include treatments in which only sperm are handled (i.e., intrauterine—or artificial—insemination) or procedures in which a woman takes

medicine only to stimulate egg production without the intention of having eggs retrieved."

Procedures are mainly fertility medication, as well as ART techniques that use more substantial and forceful interventions, of which in vitro fertilization (IVF) and expansions of it (e.g. OCR,

AZH, ICSI, ZIFT) are the most prevalent. However, there are also other manual ART, not necessarily dependent on IVF (e.g. PGD, GIFT, SSR).

Fertility medication:

Most fertility medications are agents that stimulate the development of follicles in the ovary. Examples are gonadotropins and gonadotropin releasing hormone.

Artificial insemination:

Artificial insemination involves sperm being placed into a female's uterus (intrauterine) or cervix (intracervical) using artificial means rather than by sexual intercourse. This can be a very low-tech process, performed at home by the woman alone or with her partner.

In Vitro Fertilization:

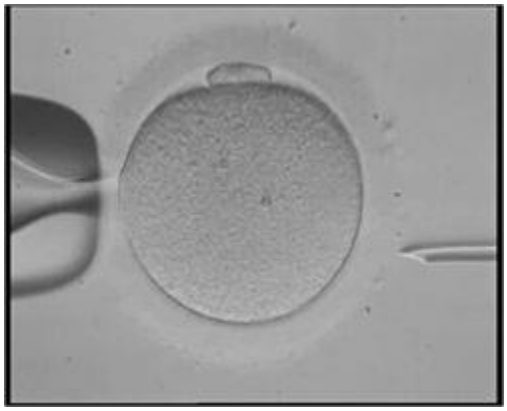
In vitro fertilization is the technique of letting fertilization of the male and female gametes (sperm and egg) occur outside the female body.

Techniques usually used in in vitro fertilization include:

- Transvaginal ovum retrieval (OCR) is the process whereby a small needle is inserted through the back of the vagina and guided via ultrasound into the ovarian follicles to collect the fluid that contains the eggs.
- Embryo transfer is the step in the process whereby one or several embryos are placed into the uterus of the female with the intent to establish a pregnancy.

Less commonly used techniques in in vitro fertilization are:

- Assisted zona hatching (AZH) is performed shortly before the embryo is transferred to the uterus. A small opening is made in the outer layer surrounding the egg in order to help the embryo hatch out and aid in the implantation process of the growing embryo.

- 

Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection (ICSI) is beneficial in the case of male factor infertility where sperm counts are very low or failed fertilization occurred with previous IVF attempt(s). The ICSI procedure involves a single sperm carefully injected into the center of an egg using a microneedle. With ICSI, only one sperm per egg is needed. Without ICSI, you need between 50,000 and 100,000.[2] This method is also sometimes employed when donor sperm is used.

- Autologous endometrial coculture is a possible treatment for patients who have failed previous IVF attempts or who have poor embryo quality. The patient's fertilized eggs are placed on top of a layer of cells from the patient's own uterine lining, creating a more natural environment for embryo development.

- In zygote intrafallopian transfer (ZIFT), egg cells are removed from the woman's ovaries and fertilized in the laboratory; the resulting zygote is then placed into the fallopian tube.

- Cytoplasmic transfer is the technique in which the contents of a fertile egg from a donor are injected into the infertile egg of the patient along with the sperm.

- Egg donors are resources for women with no eggs due to surgery, chemotherapy, or genetic causes; or with poor egg quality, previously unsuccessful IVF cycles or advanced maternal age. In the egg donor process, eggs are retrieved from a donor's ovaries,

fertilized in the laboratory with the sperm from the recipient's partner, and the resulting healthy embryos are returned to the recipient's uterus.

- Sperm donation may provide the source for the sperm used in IVF procedures where the male partner produces no sperm or has an inheritable disease, or where the woman being treated has no male partner.
- Preimplantation genetic diagnosis (PGD) involves the use of genetic screening mechanisms such as fluorescent in-situ hybridization (FISH) or comparative genomic hybridization (CGH) to help identify genetically abnormal embryos and improve healthy outcomes.
- Embryo splitting can be used for twinning to increase the number of available embryos.

Surrogacy

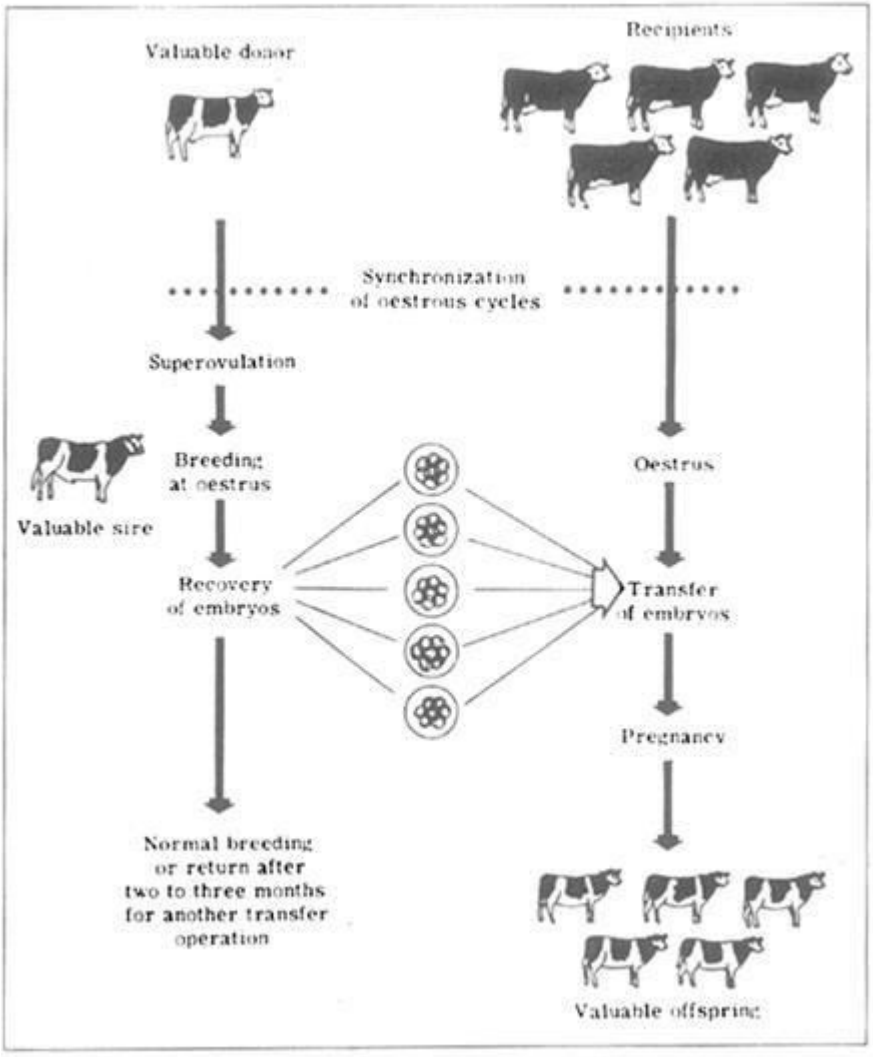
Surrogacy, where a woman agrees to become pregnant and deliver a child for a contracted party. It may be her own genetic child, or a child conceived through natural insemination, in vitro fertilization or embryo transfer using another woman's ova. Surrogacy via a gestational carrier is an option when a patient's medical condition prevents a safe pregnancy, when a patient has ovaries but no uterus due to congenital absence or previous surgical removal, and where a patient has no ovaries and is also unable to carry a pregnancy to full term.

EMBRYO TRANSFER

Embryo transfer refers to a step in the process of assisted reproduction in which embryos are placed into the uterus of a female with the intent to establish a pregnancy. This technique (which is often used in connection with in vitro fertilization (IVF)), may be used in humans or in animals, in which situations the goals may vary.

The donor cow is stimulated with a hormone (generally pregnant mare's serum gonadotrophin (PMSG) or follicle-stimulating hormone). Following such stimulation the animal comes on heat and she is bred or artificially inseminated with semen of the breeder's choice. In order to obtain maximum fertility two breedings are generally employed with a 12–hour interval, and a higher dose of semen is used the second time. It is expected that the donor will release a number of ova (10 to 15 or more) at this oestrus instead of the usual one. The ova are naturally fertilized within

the donor and proceed to develop. If these embryos are left to grow in the donor cow, resorption and or multiple pregnancies may result which are undesirable in cattle because of resulting complications and the possible occurrence of freemartins. About four to five days after breeding (when the embryos are freely "floating" within the uterus and are not yet implanted), the embryos are flushed out with a suitable biological medium.



Embryos are transferred within four to five hours of recovery; during this time they are stored with minimum changes of temperature and pH.It is important that the oestrous cycle of the recipient animal be synchronous with that of the donor (both donor and recipients should be in heat on the same day).

IN VITRO FERTILIZATION OR FERTILISATION (IVF)

***In vitro* fertilization** or **fertilisation** (**IVF**) is a process by which an egg is fertilised by sperm outside the body: *in vitro* ("in glass"). The process involves monitoring and stimulating a woman's ovulatory process, removing an ovum or ova (egg or eggs) from the woman's ovaries and letting sperm fertilise them in a liquid in a laboratory. The fertilised egg (zygote) is cultured for 2–6 days in a growth medium and is then implanted in the same or another woman's uterus, with the intention of establishing a successfulpregnancy.

IVF techniques can be used in different types of situations. It is a technique of assisted reproductive technology for treatment ofinfertility. IVF techniques are also employed in gestational surrogacy, in which case the fertilised egg is implanted into a surrogate's uterus, and the resulting child is genetically unrelated to the surrogate. In some situations, donated eggs or sperms may be used. Some countries ban or otherwise regulate the availability of IVF treatment, giving rise to fertility tourism. Restrictions on availability of IVF include to single females, to lesbians and to surrogacy arrangements. Due to the costs of the procedure, IVF is mostly attempted only after less expensive options have failed.

The first successful birth of a "test tube baby", Louise Brown, occurred in 1978. Louise Brown was born as a result of natural cycle IVF where no stimulation was made. Robert G. Edwards, the physiologist who developed the treatment, was awarded the Nobel Prize in Physiology or Medicine in 2010. With egg donation and IVF, women who are past their reproductive years or menopausecan still become pregnant. Adriana Iliescu held the record as the oldest woman to give birth using IVF and donated egg, when she gave birth in 2004 at the age of 66, a record passed in 2006. After the IVF treatment many couples are able to get pregnant without any fertility treatments.

IVF is usually used for:

- Advanced age of the woman (advanced maternal age)

- Damaged or blocked fallopian tubes (can be caused by pelvic inflammatory disease or prior reproductive surgery)
- Endometriosis
- Male factor infertility, including decreased sperm count and blockage
- Unexplained infertility (An unexplained fertility problem)
- Low sperm counts
- Problems with ovulation
- Antibody problems that harm sperm or eggs
- The inability of sperm to penetrate or survive in the cervical mucus

This type of fertilization treatment presents some **advantages**. For instance, it reduces the numbers of surgery by half because women who resort to this technique don't have to undergo surgery on their fallopian tubes. It's also a great solution for women getting in age who really want to have a baby instead of their physical condition.

However, IVF is usually more reserved for women under 35 to eliminate a certain number of risks associate with advanced maternal age. In addition, this method is considered as safe as any other one. A recent study covered nearly 1,000 children conceived through these methods in 5 European countries and found that the children, monitored from birth to 5 years of age, were as healthy as children conceived naturally.

According to the Society of Assisted Reproductive Technologies (SART), **the approximate chance of giving birth to a live baby after IVF** is as follows:

- 41-43% for women under age 35
- 33-36% for women age 35 – 37
- 23-27% for women ages 38 – 40
- 13-18% for women over age 41

The in vitro fertilization generally goes through five (5) basics steps:

Step 1 : Stimulation or super ovulation

Fertility drugs are given to the woman in order to boost her egg production. Effectively, during an ordinary month, only a single egg might mature. With this type of hormone treatment, her ovaries are stimulated and produced a large number of mature eggs, sometimes more than 20.

Step 2 : Egg retrieval

This step starts with a minor surgery, sometimes substituted for laparoscopic surgery, called follicular aspiration. Taking place in the doctor's office without general anaesthesia, this technique allows doctors to remove eggs from the woman's ovary by inserting a thin needle through the vagina and into the ovary and sacs (follicles) containing the eggs. This needle is connected to a suction device, pulling the eggs out of the ovary one at the time.

Step 3 : Insemination and Fertilization

During this time, the man has to provide a semen sample. The sperm are separated from the semen in a laboratory procedure. Then, the man's sperms are placed with the best quality from the woman's eggs in an environmentally controlled chamber. This step is what we call insemination. Generally, the sperm fertilizes an egg a few hours after the insemination. However, if this step doesn't work, the sperm has to be injected directly into the egg, it's the intracytoplasmic sperm injection (ICSI).

Step 4 : Embryo culture

After the fertilization (about 18 hours later), the egg will divide to become an embryo (so we can determine if the last step worked or not). During this step, laboratory staff regularly check the embryo to make sure that it grows up properly. Within around five (5) days, a normal embryo has several cells that are actively dividing. These embryos are incubated and observed over 2-3 days or longer.

Step 5 : Embryo transfer

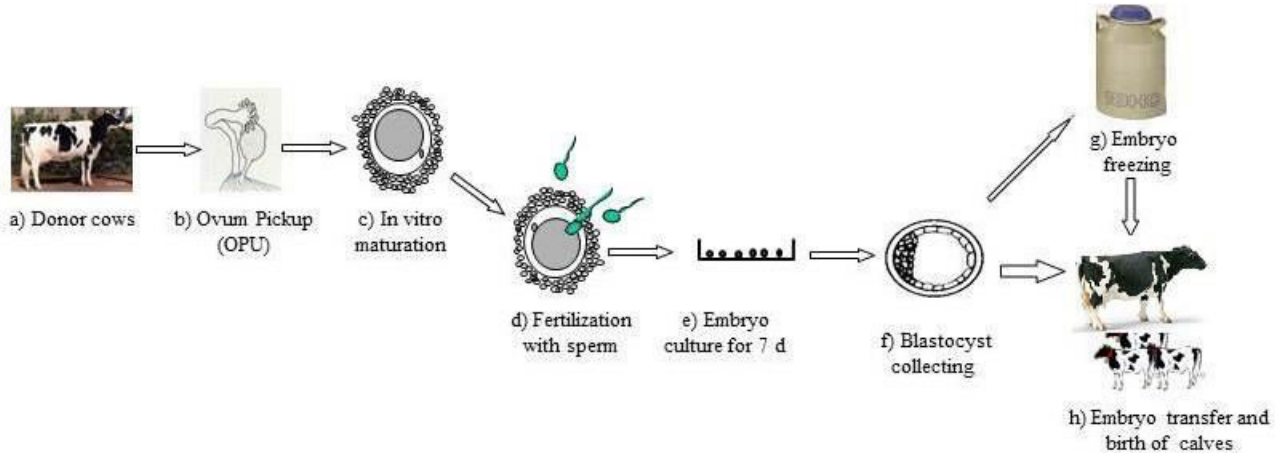
After these few days of embryos development observations, they are introduced into the woman's womb through the cervix with a catheter (a long slender tube) containing the precious embryos. If an embryo sticks to (implants) in the lining of the womb and grows, pregnancy

results. The number of embryos implanted depends on several factors such as the age of the mother or the country where the treatment is giving. However, North American Doctors agree to the fact that no more than two (2) embryos should be implanted at a time to eliminate multiple births which are more dangerous than beneficial. Also, unused embryos may be frozen and implanted or donated at a later date.

Main advantages of IVF in livestock market

- IVF makes it possible to rapidly multiply and breed genetically superior cattle within a short generation interval. Multiplying pre-selected females.
- Increasing efficiency of multiplication in breeding, permitting determination of sex of the offspring and pre-testing of actual fertility status of the bull.
- Genetics selection based on male and female.
- Productivity: Raise of average litre per cow.
- Improve pregnancy rates in lactating cows.
- We guarantee 100% pregnancy rate.
- Lower costs: Importation of live dairy cattle can cost P150,000 to P180,000 per animal initially.
- No quarantine needed: animals are born in the country.
- Potential to create a local raze and market it worldwide: beef and dairy cow.
- No need to super ovulate the cow, no need of hormones like with AI.
- Highly efficient use of semen in comparison with other repro tools (AI). One straw of conventional frozen semen can fertilized a high number of female donors (>25)..
- Genetic Jump: no need to wait too long to improve genetics.
- Rescue genetics: produce embryos from females in different physiologic conditions (terminal, disease, culled, accident, etc.)

- Proven Technology: All Latin America, USA, Europe, South Africa, Australia, China, Philippines will be the first one in South East Asia.
- Genetics generated in-house in the Philippines: money reinvested in the country.



EMBRYO CLONING

The technique to create **cloned embryos** is similar to that used to create Dolly the sheep, the world's first mammal **cloned** from a single adult cell. Scientists extract DNA from the cell of an adult patient and insert it into a hollowed out donor egg.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT), also called nuclear transfer, uses a different approach than artificial embryo twinning, but it produces the same result: an exact genetic copy, or clone, of an individual. This was the method used to create Dolly the Sheep.

What does SCNT mean? Let's take it apart:

Somatic cell: A somatic cell is any cell in the body other than sperm and egg, the two types of reproductive cells. Reproductive cells are also called germ cells. In mammals, every somatic cell has two complete sets of chromosomes, whereas the germ cells have only one complete set.

Nuclear: The nucleus is a compartment that holds the cell's DNA. The DNA is divided into packages called chromosomes, and it contains all the information needed to form an organism. It's small differences in our DNA that make each of us unique.

Transfer: Moving an object from one place to another. To make Dolly, researchers isolated a **somatic cell** from an adult female sheep. Next they removed the nucleus and all of its DNA from an egg cell. Then they **transferred**the **nucleus** from the somatic cell to the egg cell. After a couple of chemical tweaks, the egg cell, with its new nucleus, was behaving just like a freshly fertilized egg. It developed into an embryo, which was implanted into a surrogate mother and carried to term. (The transfer step is most often done using an electrical current to fuse the membranes of the egg and the somatic cell.)

The lamb, Dolly, was an exact genetic replica of the adult female sheep that donated the somatic cell. She was the first-ever mammal to be cloned from an adult somatic cell.

Watch these videos of enucleation and nuclear transfer.

Gene Cloning

Gene cloning, also known as Molecular cloning, refers to the process of making multiple molecules. Cloning is commonly used to amplify DNA fragments containing whole genes, but it can also be used to amplify any DNA sequence such as promoters, non-coding sequences and randomly fragmented DNA. It is used in a wide array of biological experiments and practical applications ranging from genetic fingerprinting to large scale protein production. To amplify any DNA sequence in a living organism, that sequence must be linked to an origin of replication, which is a sequence of DNA capable of directing the propagation of itself and any linked sequence. However, a number of other features are needed and a variety of specialized cloning vectors (small piece of DNA into which a foreign DNA fragment can be inserted) exist that allow protein expression, tagging, single stranded RNA and DNA production and a host of other manipulations.

Cloning of any DNA fragment essentially involves four steps

1. fragmentation - breaking apart a strand of DNA

2. ligation - gluing together pieces of DNA in a desired sequence

3. transfection - inserting the newly formed pieces of DNA into cells

4. screening/selection - selecting out the cells that were successfully transfected with the new DNA

Although these steps are invariable among cloning procedures a number of alternative routes can be selected, these are summarized as a 'cloning strategy'.

Therapeutic Cloning

Somatic-cell nuclear transfer, known as SCNT, can also be used to create embryos for research or therapeutic purposes. The most likely purpose for this is to produce embryos for use in stem cell research. This process is also called "research cloning" or "therapeutic cloning." The goal is not to create cloned human beings (called "reproductive cloning"), but rather to harvest stem cells that can be used to study human development and to potentially treat disease. While a clonal human blastocyst has been created, stem cell lines are yet to be isolated from a clonal source.

Therapeutic cloning is achieved by creating embryonic stem cells in the hopes of treating diseases such as diabetes and Alzheimer's. The process begins by taking out the nucleus (containing the DNA) from an egg cell and putting in it a nucleus from the adult cell to be cloned. In the case of someone with Alzheimer's disease, the nucleus from a skin cell of that patient is placed into an empty egg. The reprogrammed cell begins to develop into an embryo because the egg reacts with the transferred nucleus. The embryo will become genetically identical to the patient. The embryo will then form a blastocyst which has the potential to form/become any cell in the body.

In SCNT, not all of the donor cell's genetic information is transferred, as the donor cell's mitochondria that contain their own mitochondrial DNA are left behind. The resulting hybrid cells retain those mitochondrial structures which originally belonged to the egg. As a

consequence, clones such as Dolly that are born from SCNT are not perfect copies of the donor of the nucleus.

Reproductive Cloning

Reproductive cloning (also known as Organism cloning) refers to the procedure of creating a new multicellular organism, genetically identical to another. In essence this form of cloning is an asexual method of reproduction, where fertilization or inter-gamete contact does not take place. Asexual reproduction is a naturally occurring phenomenon in many species, including most plants (vegetative reproduction) and some insects. Scientists have made some major achievements with cloning, including the asexual reproduction of sheep and cows.

Reproductive cloning generally uses "somatic cell nuclear transfer" (SCNT) to create animals that are genetically identical. This process entails the transfer of a nucleus from a donor adult cell (somatic cell) to an egg that has no nucleus. If the egg begins to divide normally it is transferred into the uterus of the surrogate mother. Such clones are not strictly identical since the somatic cells may contain mutations in their nuclear DNA.

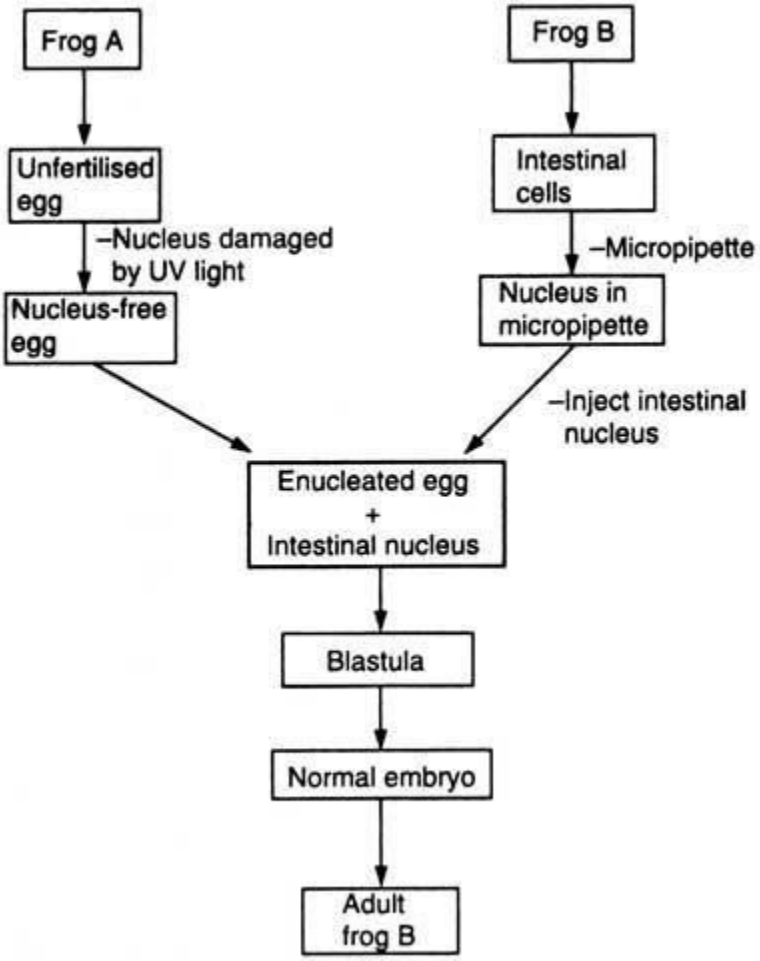
Artificial embryo splitting or embryo twinning may also be used as a method of cloning, where an embryo is split in the maturation before embryo transfer. It is optimally performed at the 6- to 8-cell stage, where it can be used as an expansion of IVF to increase the number of available embryos. If both embryos are successful, it gives rise to monozygotic (identical) twins.

NUCLEAR TRANSFER:

A clone is a population of cells or organisms derived asexually from a single ancestor. They are genetically identical to each other to their common ancestor. Cloning means the production of exact genetic replica copies of an individual. They can not be considered as an offspring but simply the copy of a given individual. Much work has been done on cloning in plants and microorganisms. However, the techniques used in plants can not be applied for animals. Moreover, many animals from a single genetically superior embryo can be produced. Still there is no method of finding out which embryos are capable of cloning. It is useless to clone an embryo if it is not superior.

Nuclear transplantation (also nuclear transfer) involves removal of a single blastomere from a cleavage stage embryo with a fine micropipette of glass, and placing it under the outer membrane of an unfertilized mature enucleated oocyte (whose haploid nucleus has been removed by using micropipette or destroyed by UV light). For the first time in 1955, Robert Briggs and Tom King at Cancer Research Institute, Philadelphia (USA) carried out nuclear transplantation experiment on embryonic cells of frog. They transferred nucleus of undifferentiated blastula (a stage soon after fertilization of egg) into an enucleated egg cell. They noticed the normal development of the embryo. When they performed serial transplantation of differentiated nucleus from late gastrula (a stage after blastula) into a nucleus-free unfertilized egg, abnormal embryos were formed. This shows that cell nucleus is differentiated with embryo development. In 1960s, J.B. Gurdon at Oxford University, U.K. transferred differentiated intestinal nucleus of a frog into nucleus-free unfertilized egg of different amphibian species*(Xenopuslaevis).* The embryo developed into tadpole and matured into frog (Gurdon, 1962). This new enucleated cell developed into normal embryo. Any damage to the donor nucleus during transplantation leads to abnormal development .

JOHN GORDAN"S EXPERIMENT:



- **DOLLY** - The first mammalian clone. 'Dolly', the worlds' first mammalian clone has been created from a fully differentiated non-reproductive cell of an adult sheep. It was born in February, 1996. The name Dolly has been given after an American country singer, Dolly Parton. In 1995, Ian Wilmut and his team of researchers at Roslin Institute, Edinburgh, Scotland, took udder (a fully differentiated tissue) from six year old sheep, Fin Dorset Ewe, and placed it in special solution that controlled cell cycle of cell division. The cell was deprived off certain nutrient. At the same time an unfertilized egg

was obtained from another adult sheep .Its nucleus was carefully removed leaving the intact cytoplasm in egg. The nucleus of udder cell was taken out and transferred into nucleus-free egg. This was facilitated by applying mild electric sock. The newly transplanted nucleus soon became functional according to the new cytoplasm in which it had been artificially transferred. This viable combination underwent cleavage like normal zygote. This so called embryo was then transplanted into the uterus of a third adult sheep (surrogate mother/foster mother) for its further development.

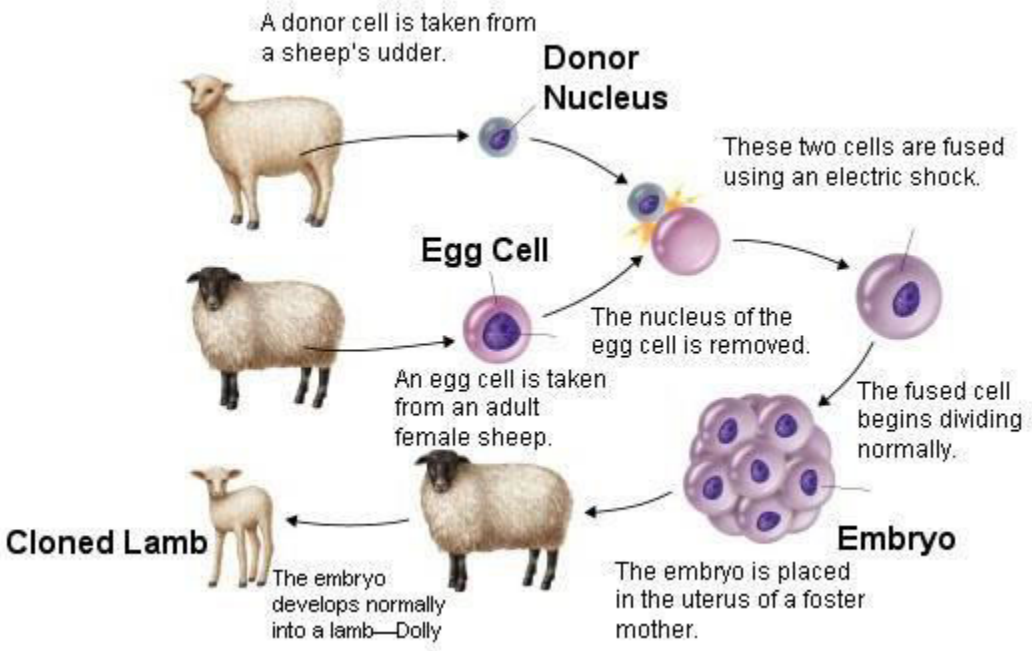
Finally, a normal healthy little lamb, Dolly was born in February, 1996 which was genetically similar to the *clone mother* from which nuclear DNA was taken out. It does not have any similarity with that sheep from which egg was taken out or surrogate mother because they did not contribute any genetic character (Wimut*et al.,* 1997). Thus, Dolly has only a single parent because she has born asexually, a characteristic feature found in lower forms of animal life, not in mammals.

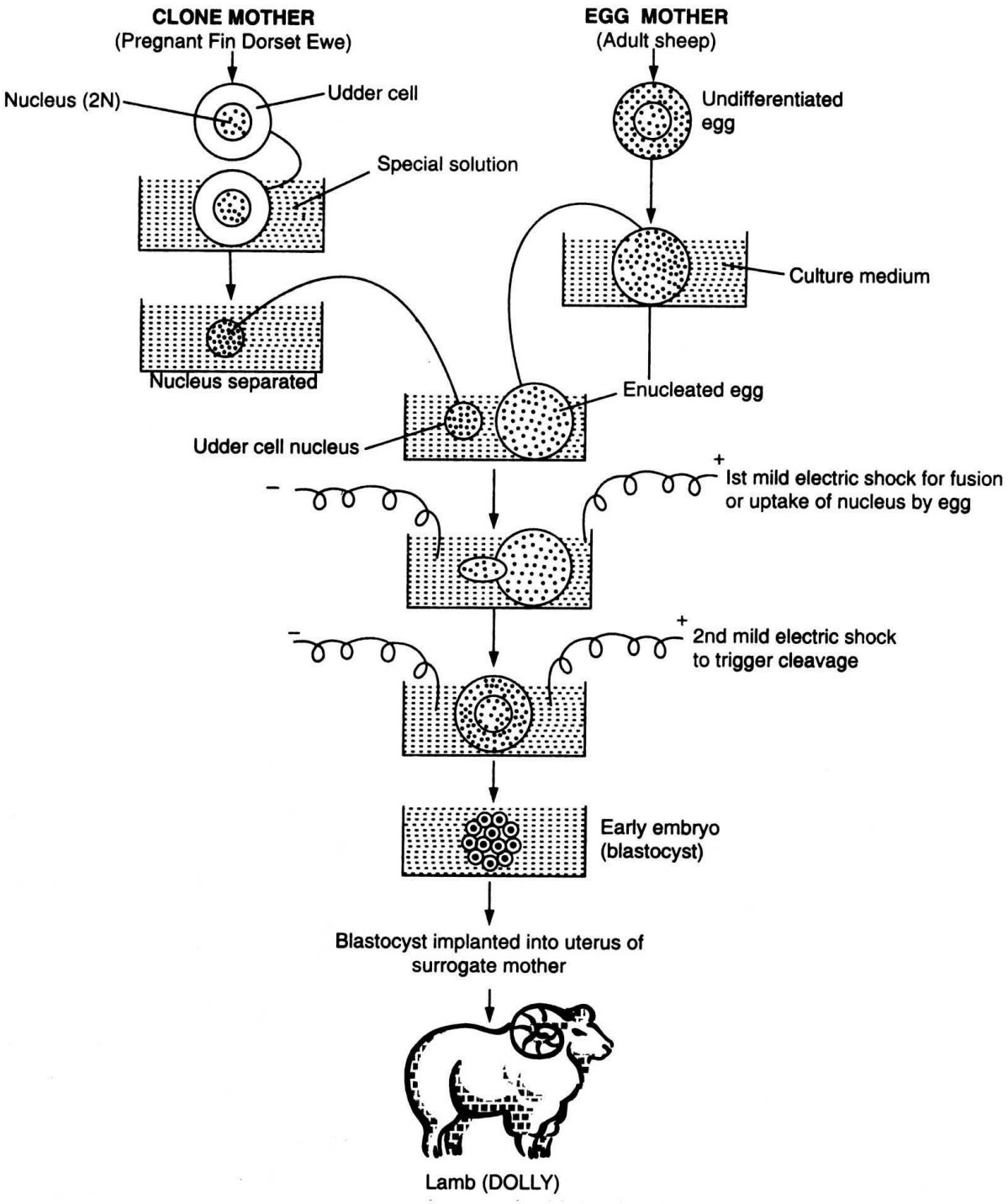
Although behind this great success the rate of success is very slow, yet it has given some hope to embryo-biotechnologists to bring about refinement. Out of 277 nuclei transferred singly to enucleated egg, only 29 eggs grew into embryos. Out of these, only 13 embryo could be successfully transplanted into surrogate mothers. Of these only one ewe was successful in giving birth to an offspring, Dolly (Wilmut *et al,* 1996).

The significant considerations that can be derived from this experiment are that:*(i)* the genes of differentiated cells have inherent totipotency, *(ii)* the interplay between the regulatory system of the genome in nucleus and the cytoplasmic factors of egg may make a cell totipotent, *(iii)* possibly the cytoplasm of enucleated egg makes the transplanted nucleus totipotent like

that of normal fertilized egg nucleus, *(iv)* the maternally derived information in egg cytoplasm has an important role in cleavage which usually occurs after fertilization.

Hence, it is the egg cytoplasm but not the nucleus that regulates cleavage. Because the udder cell nucleus has limited potential for mitosis; it is the egg cytoplasm that interacted intracellularly with udder cell nucleus and stimulated to undergo repeated mitosis, *(v)* carbon copy of the adult sheep could be produced without involving sperms from male partner, and *(vi)*the cloned animal produced via nuclear transplantation technique will be capable of restoring fertility as in 1998 Dolly gave birth to a little lamb named *Bonny.*




CLONE MOTHER
(Pregnant Fin Dorset Ewe)
EGG MOTHER
(Adult sheep)
Nucleus (2N)
Udder cell
Undifferentiated egg
Special solution
Culture medium
Nucleus separated
Enucleated egg
Udder cell nucleus
+
Ist mild electric shock for fusion or uptake of nucleus by egg
–
+
2nd mild electric shock to trigger cleavage
–
Early embryo (blastocyst)
Blastocyst implanted into uterus of surrogate mother
Lamb (DOLLY)

Potential Applications of Cloning

- SCNT is seen as a good method for producing agriculture animals for food consumption. It has successfully cloned sheep, cattle, goats, and pigs.
- Cloning is seen as a solution to the endangered species that are on the verge of going extinct.
- Cloning technologies are used widely in plant conservation.
- Reproductive cloning may enable researchers to make copies of animals with the potential benefits for the fields of medicine and agriculture for testing new drugs and treatment strategies.
- Embryonic stem cells which have the unique ability to generate virtually all types of cells in an organism can be used to grow tissues in the laboratory through therapeutic cloning that can be used to grow healthy tissue to replace injured or diseased tissues.
- It may be possible to learn more about the molecular causes of disease by studying embryonic stem cell lines from cloned embryos derived from the cells of animals or humans with different diseases.

Potential Drawbacks of Cloning

- Many conservation biologists and environmentalists vehemently oppose cloning endangered species—mainly because they think it may deter donations to help preserve natural habitat and wild animal populations. The "rule-of-thumb" in animal conservation is that, if it is still feasible to conserve habitat and viable wild populations, breeding in captivity should not be undertaken in isolation.
- In a 2006 review, David Ehrenfeld concluded that cloning in animal conservation is an experimental technology that, at its state in 2006, could not be expected to work except

by pure chance and utterly failed a cost-benefit analysis.

- The human consumption of meat and other products from cloned animals was approved by the FDA on December 28, 2006, with no special labeling required. Such practice has met strong resistance in other regions, such as Europe, particularly over the labeling issue.
- Cloning from a single specimen could not create a viable breeding population in sexually reproducing animals.
- If males and females were to be cloned, the question would remain open whether they would be viable at all in the absence of parents that could teach or show them their natural behavior.
- As the procedure currently cannot be automated, and has to be performed manually under a microscope, SCNT is very resource intensive.
- The biochemistry involved in reprogramming the differentiated somatic cell nucleus and activating the recipient egg is far from being well-understood.
- The mitochondria in the cytoplasm also contains DNA and during SCNT this mitochondrial DNA is wholly from the cytoplasmic donor's egg, thus the mitochondrial genome is not the same as that of the nucleus donor cell from which it was produced. This may have important implications for cross-species nuclear transfer in which nuclear- mitochondrial incompatibilities may lead to death.
- Many religious organizations oppose all forms of cloning, on the grounds that life begins at conception.
- Researchers have found several abnormalities in cloned organisms. The cloned organism may be born normal and resemble its non-cloned counterpart, but majority of the time will express changes in its genome later on in life.

QUADRIPARENTAL-HYBRIDS:

During 1960s, Beatrice Mintz at Cancer Research Institute, Philadelphia (USA)

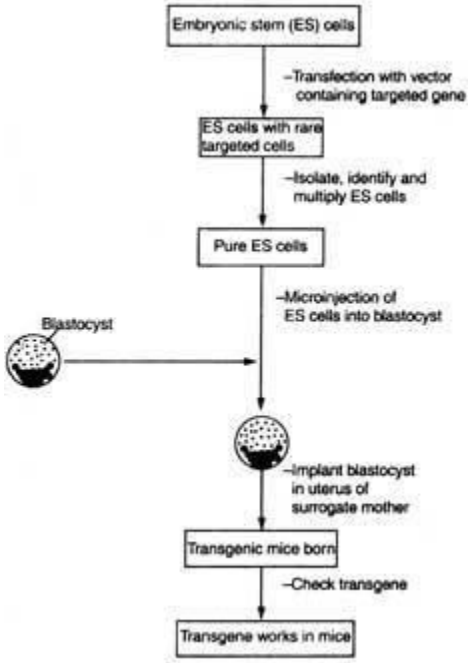
demonstrated the interesting experimentation. She carried out fusion of embryos of two

different species of mouse. This resulted in the formation of a single embryo which finally developed into a normal healthy animal having four parents. Embryo A was derived from the cross of male x female of one species, and embryo B derived after cross of male x female of the other species. The embryo A and B were united together and produced a single mass. In this experiment Mintz removed zonapellucida membrane of two early embryos and placed them in a suitable culture medium. The embryonic cells of the two embryos of blastula stage united randomly into a single mass of double sized embryo (blastocyst). A fresh membrane developed around the embryo. Then the embryo was transferred into the uterus of a foster mother. The foster mother was mated with a sterile male to bring her into proper stage for implantation. The first offspring with four parents was born in 1965. Similarly, exciting experiments have been done on another mammal. Following the same technique a hybrid of goat and sheep named geep was produced (Joshi, 1998).

- At present two types of techniques for embryo cloning viz., nuclear transplantation (transfer) and embryonic stem cells, are being developed.

Embryonic stem cells (**ES cells**) are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage preimplantation embryo. Human embryos reach the blastocyst stage 4–5 days post fertilization, at which time they consist of 50–150 cells. Isolating the embryoblast or inner cell mass (ICM) results in destruction of the blastocyst, which raises ethical issues, including whether or not embryos at the pre-implantation stage should be considered to have the same moral or legal status as more developed human beings.

- Human ES cells measure approximately 14 µm while mouse ES cells are closer to 8 µm.



Embryonic Stem (ES) cells

- Cloning of mice could not be done as in sheep via nuclear transplantation. This was due to acceleration of developmental programmes of embryo. However, it is evident that before first embryonic division the cell has started its process of differentiation. Therefore, for cloning of mice an alternative approach has been made *i.e.* the use of ES cells. A blastocyst of mouse is place in culture condition. The inner cells that form future foetus continue to divide and remain in undifferentiated totipotent state as ES cells. There is a peptide growth factor known as leukaemia inhibitory factor (LIF) which establishes and maintains ES cell lines. The ES cell lines will be very useful in the area of production of transgenic animals *(see* preceding sections). However, the ES cells are used in two different ways: a small number of ES cells can be injected into blastocoel space of a blastocyst (Fig. 7.5). The ES cells get mixed with inner mass of cells of blastocyst to produce a chimera mouse which is a mixture of two cell genotype having the patches of different colored fur. Crossing of male and female chimera will allow selection of

  homozygous mice derived from ES cells (Read and Smith, 1996).

Pluripotent

Embryonic stem cells of the inner cell mass are pluripotent, that is, they are able to differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body. Pluripotency distinguishes embryonic stem cells from adult stem cells found in adults; while embryonic stem cells can generate all cell types in the body, adult stem cells are multipotent and can produce only a limited number of cell types. If the pluripotent differentiation potential of embryonic stem cells could be harnessed in vitro, it might be a means of deriving cell or tissue types virtually to order. This would provide a radical new treatment approach to a wide variety of conditions where age, disease, or trauma has led to tissue damage or dysfunction.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – V– ANIMAL BIOTECHNOLOGY – SBT1305

UNIT V TECHNOLOGY –

APPLICATIONS OF ANIMAL BIOTECHNOLOGY

Hybridoma Technology-Uses of monoclonal antibodies, DNA fingerprinting & RFLP in Domestic animals. Gene theraphy-Techniques & Developments and future prespective Applications of Biotechnolgy in Medicine, Environmental biotechnology and Agriculture. IPR & Biosafety

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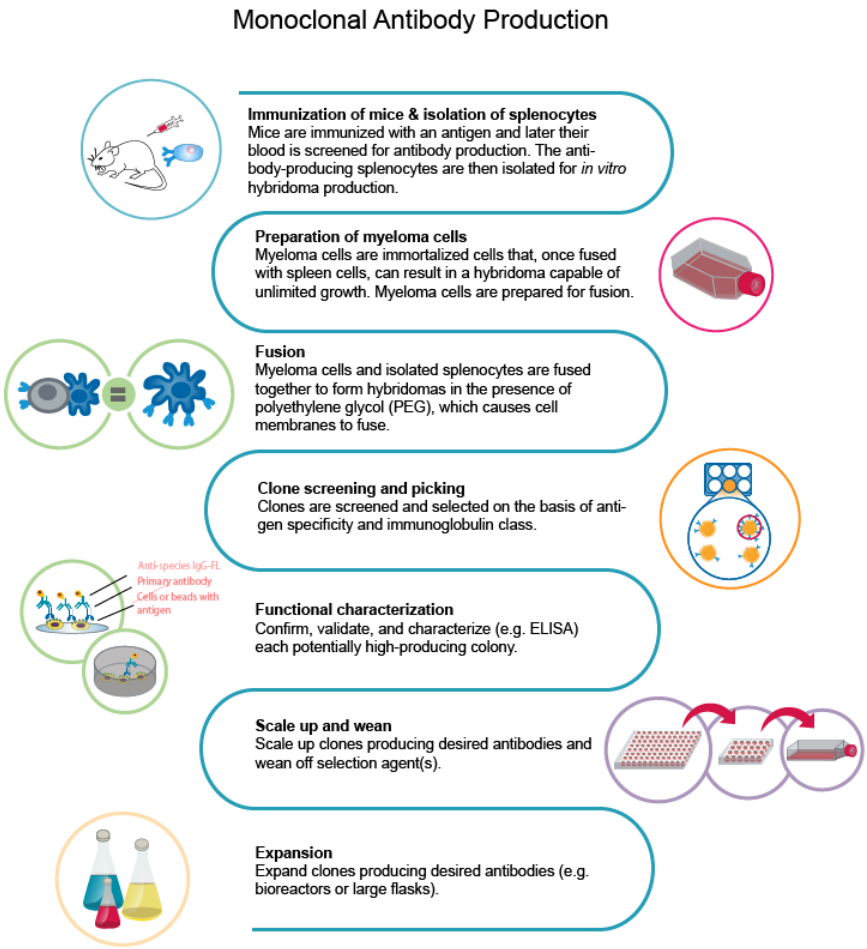

Diagnostic Applications:

Monoclonal antibodies have revolutionized the laboratory diagnosis of various diseases. For this purpose, MAbs may be employed as diagnostic reagents for biochemical analysis or as tools for diagnostic imaging of diseases.

(A) MAbs in Biochemical Analysis:

Diagnostic tests based on the use of MAbs as reagents are routinely used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) in the laboratory. These assays measure the circulating concentrations of hormones (insulin, human chorionic gonadotropin, growth hormone, progesterone, thyroxine, triiodothyronine, thyroid stimulating hormone, gastrin, renin), and several other tissue and cell products (blood group antigens, blood clotting factors, interferon's, interleukins, histocompatibility antigens, tumor markers). In recent years, a number of diagnostic kits using MAbs have become commercially available. For instance, it is now possible to do the early diagnosis of the following conditions/diseases.

Pregnancy:Pregnancy by detecting the urinary levels of human chorionic gonadotropin.

Cancers:Cancers estimation of plasma carcinoembryonic antigen in colorectal cancer, and prostate specific antigen for prostate cancer. Besides diagnosis, estimation of tumor markers is also useful for the prognosis of cancers. That is a gradual fall in a specific tumor marker is observed with a reduction in tumor size, following treatment.

Hormonal disorders:Hormonal disorders analysis of thyroxine, triiodothyronine and thyroid stimulating hormone for thyroid disorders.

Infectious diseases: Infectious diseases by detecting the circulatory levels of antigens specific to the infectious agent e.g., antigens of Neisseria gonorrhoeae and herpes simplex virus for the diagnosis of sexually transmitted diseases.

(B) MAbs in Diagnostic Imaging: Radiolabeled—MAbs are used in the diagnostic imaging of diseases, and this technique is referred to as immunoscintigraphy. The radioisotopes commonly used for labeling MAb are iodine—131 and technetium—99. The MAb tagged with radioisotope are injected intravenously into the patients.

These MAbs localize at specific sites (say a tumor) which can be detected by imaging the radioactivity. In recent years, single photon emission computed tomography (SPECT) cameras are used to give a more sensitive three dimensional appearance of the spots localized by radiolabeled—MAbs.

Immunoscintigraphy is a better diagnostic tool than the other imaging techniques such as CT scan, ultrasound scan and magnetic resonance. For instance, immunoscintigraphy can differentiate between cancerous and non-cancerous growth, since radiolabeled—MAbs are tumor specific. This is not possible with other imaging techniques. Monoclonal antibodies are successfully used in the diagnostic imaging of cardiovascular diseases, cancers and sites of bacterial infections.

Cardiovascular diseases:

Myocardial infarction:

The cardiac protein myosin gets exposed wherever myocardial necrosis (death of cardiac cells) occurs. Antimyosin MAb labeled with radioisotope indium chloride (111 In) is used for detecting myosin and thus the site of myocardial infarction. Imaging of radiolabeled MAb, is usually done after 24-48 hours of intravenous administration.

This is carried out either by planner gamma camera or single photon emission computed tomography (SPECT). It is possible to detect the location and the degree of damage to the heart by using radiolabeled antimyosin MAb. Thus, this technique is useful for the diagnosis of heart attacks.

Deep vein thrombosis (DVT):

DVT refers to the formation of blood clots (thrombus) within the blood veins, primarily in the lower extremities. For the detection of DVT, radioisotope labeled MAb directed against fibrin or platelets

can be used. The imaging is usually done after 4 hours of injection. Fibrin specific MAbs are successfully used for the detection of clots in thigh, pelvis, calf and knee regions.

Atherosclerosis:

Thickening and loss of elasticity of arterial walls is referred to as atherosclerosis. Atherosclerotic plaques cause diseases of coronary and peripheral arteries. Atherosclerosis has been implicated in the development of heart diseases. MAb tagged with a radiolabel directed against activated platelets can be used to localize the atherosclerotic lesions by imaging technique.

Cancers:

Monoclonal antibodies against many types of human cancers are now available. A selected list of tumor markers (along with the associated cancers) that can be used for MAb imaging is given in Table 17.2. Tumors can be located in patients using radioisotope labeled MAbs specific to the protein(s), particularly of membrane origin.

TABLE 17.2 Selected tumor markers along with the associated cancers used in MAb imaging

Tumor marker	*Associated cancer(s)*
Carcinoembryonic antigen (CEA)	Cancers of colon, stomach, pancreas
Alpha fetoprotein	Cancers of liver, and germ cells of testes
Human chorionic gonadotropin	Choriocarcinoma
Prostatic acid phosphatase	Prostate cancer
Epiderminal growth factor receptor	Melanoma
Tumor—associated cell surface antigens	Various cancers

It has been possible to detect certain cancers at early stages (lung cancer, breast cancer, ovariran cancer, malanoma, colorectal cancer) by employing MAbs. About 80 per cent specificity has been achieved for detecting cancers by this approach.

An iodine (131l) labeled monoclonal antibody specific to breast cancer cells when administered to the patients detects (by imaging) the spread of cancer (metastasis) to other regions of the body. This is not possible by scanning techniques.

The imaging technique by using MAb can also be used to monitor therapeutic responses of a cancer. There are certain limitations in using MAb in cancer diagnosis and prognosis. These include the difficulty in the selection of a specific MAb and the access of MAb to the target site of the tumor which may be less vascularized.

MAbs in immunohistopathology of cancers:

The pathological changes of the cancerous tissue can be detected by immunohistochemical techniques. This can be done by using MAb against a specific antigen.

MAbs in hematopoietic malignancies:

Hematopoietic stem cells in bone marrow are the precursors for different blood cells, B- and T-lymphocytes which are produced in a stepwise transformation. During malignancy, transformation of lymphocytes stops at a particular stage of maturation. This can be detected by using stage-specific MAbs.

Bacterial infections:

In recent years, attempts are made to detect the sites of infections by using MAbs. This is made possible by directing MAb against bacterial antigens. Further, monoclonal antibodies against inflammatory leucocytes which accumulate at infection site are also useful to specifically detect localized infections.

2. Therapeutic Applications:

Monoclonal antibodies have a wide range of therapeutic applications. MAbs are used in the treatment of cancer, transplantation of bone marrow and organs, autoimmune diseases, cardiovascular diseases and infectious diseases.

The therapeutic applications of MAbs are broadly grouped into 2 types:

(A) Direct use of MAbs as therapeutic agents

(B) MAbs as targeting agents.

(A) MAbs as Direct Therapeutic Agents:

Monoclonal antibodies can be directly used for enhancing the immune function of the host. Direct use of MAbs causes minimal toxicity to the target tissues or the host.

In destroying disease-causing organisms:

MAbs promote efficient opsonization of pathogenic organisms (by coating with antibody) and enhance phagocytosis. In fact, MAbs were found to protect chimpanzees against certain viral (hepatitis B-virus) and bacterial (E. coli Haemophilus influenza, Streptococcus sp and Pseudomonas sp) infections.

In the treatment of cancer:

MAbs, against the antigens on the surface of cancer cells, are useful for the treatment of cancer. The antibodies bind to the cancer cells and destroy them. This is brought out by antibody—dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity and phagocytosis of cancer cells (coated with MAbs) by reticuloendothelial system.

The patients suffering from leukemia, colorectal cancer, lymphoma and melanoma have been treated with MAbs. However, there was a wide variation in the success rate. A monoclonal antibody specific to the cells of leukemia is used to destroy the residual leukemia cells without affecting other cells. MAbs are used in vitro to remove the residual tumor cells prior to autologous bone marrow transplantation (transplantation of the patient's own bone marrow cells, due to non-availability of a suitable donor).

Limitations for direct use of MAbs in cancer:

1. The MAbs produced in mice and directly used for therapeutic purposes may lead to the development of anti-mouse antibodies and hypersensitivity reactions.

2. All the cancer cells may not carry the same antigen for which MAb has been produced. Thus, MAbs may not be attached to some cancer cells at all.

3. The free antigens (of target cells) present in the circulation may bind to MAbs and prevent them from their action on the target cells.

In the immunosuppression of organ transplantation:

In the normal medical practice, immunosuppressive drugs such as cyclosporin and prednisone are administered to overcome the rejection of organ transplantation. In recent years, MAbs specific to T-lymphocyte surface antigens are being used for this purpose. The monoclonal antibody namely OKT3, was the first MAb to be licensed by U.S.

Food and Drug Administration for use as immunosuppressive agent after organ transplantation in humans. OKT3 specifically directed against CD3 antigen of T-lymphocytes is successfully used in renal and bone marrow transplantations. In the normal course, CD3 antigen activates T-lymphocytes and plays a key role in organ transplant rejection (destroys the foreign cells in the host). This is prevented by use of MAb against CD3 antigen.

In the treatment of AIDS:

Immunosuppression is the hall mark of AIDS. This is caused by reduction in CD4 (cluster determinant antigen 4) cells of T-lymphocytes. The human immunodeficiency virus (HIV) binds to specific receptors on CD4 cells by using surface membrane glycoprotein (gp120).

Genetic engineers have been successful to attach Fc portion of mouse monoclonal antibody to human CD4 molecule. This complex has high affinity to bind to membrane glycoprotein gp120 of virus infected cells. The Fc fragment induces cell-mediated destruction of HIV infected cells (Fig. 17.7).

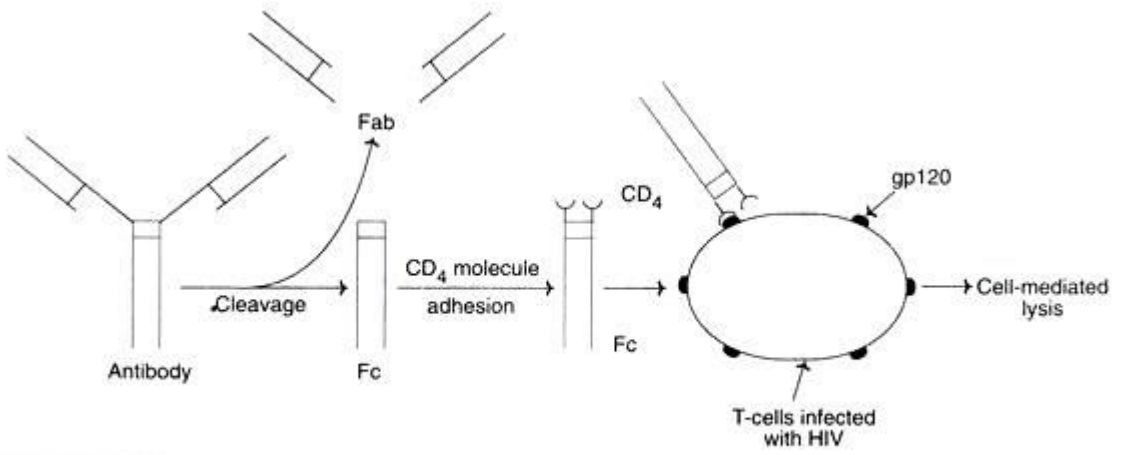

Fig. 17.7 : Modified monoclonal antibody in the treatment of AIDS.

In the treatment of autoimmune diseases:

Autoimmune diseases like rheumatoid arthritis and multiple sclerosis are of great concern. Some success has been reported in the clinical trials of rheumatoid arthritis patients by using MAbs directed against T-lymphocytes and B-lymphocytes.

(B) MAbs as Targeting Agents in Therapy:

Toxins, drugs, radioisotopes etc., can be attached or conjugated to the tissue-specific monoclonal antibodies and carried to target tissues for efficient action. This allows higher concentration of drugs to reach the desired site with minimal toxicity. In this way, MAbs are used for the appropriate delivery of drugs or isotopes.

MAbs in use as immunotoxins:

The toxins can be coupled with MAbs to form immunotoxins and used in therapy e.g., diphtheria toxin, Pseudomonas exotoxin, toxins used for cancer treatment. Anti-Tac MAb raised against IL2-R (T-cell growth factor receptor) can be conjugated with exotoxin of Pseudomonas sp. This immunotoxin can be used to destroy the malignant T-cells in the patients suffering from T-cell leukemia (Note: IL2-R is expressed in abnormal T-cells with lymphoid malignancies).

Ricin is a cytotoxic protein derived from castor oil plant. It is composed of two polypeptide chains (A and B) held together by a disulfide linkage. The B-chain of ricin binds to the cell surface. This binding facilitates the A-chain of ricin to enter the cell and inhibit the function of ribosomes (i.e. biosynthesis of all proteins is blocked).

This results in the death of cells (Fig. 17.8A). Ricin can be subjected to oxidation to separate to A and B chains. The toxic A-chain can be conjugated to MAb that is specific to cancer cells. The tumor- specific MAb bound to A-chain of ricin binds to cancer cells and not to normal cells. Once the A-chain enters the cells, it blocks ribosomal function, leading to the death of cancer cells (Fig. 17.8B).

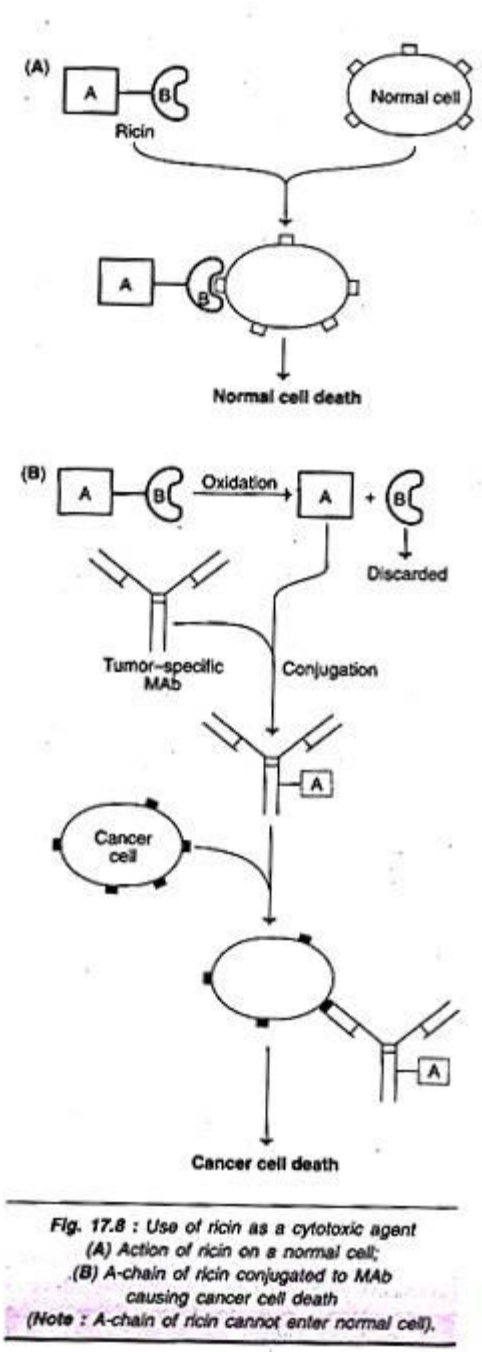

Fig. 17.8 : Use of ricin as a cytotoxic agent (A) Action of ricin on a normal cell; (B) A-chain of ricin conjugated to MAb causing cancer cell death (Note : A-chain of ricin cannot enter normal cell).

MAbs in drug delivery:

In general, the drugs are less effective in vivo (in the living body) when compared to in vitro (in laboratory when tested with cultured cells). This is mainly due to the fact that sufficient quantity of

the drug does not reach the target tissue. This problem can be -solved by using tissue-specific MAbs. The drugs can be coupled with MAb (directed against a cell surface antigen of the cells, say a tumor) and specifically targeted to reach the site of action (Fig. 17.9A).

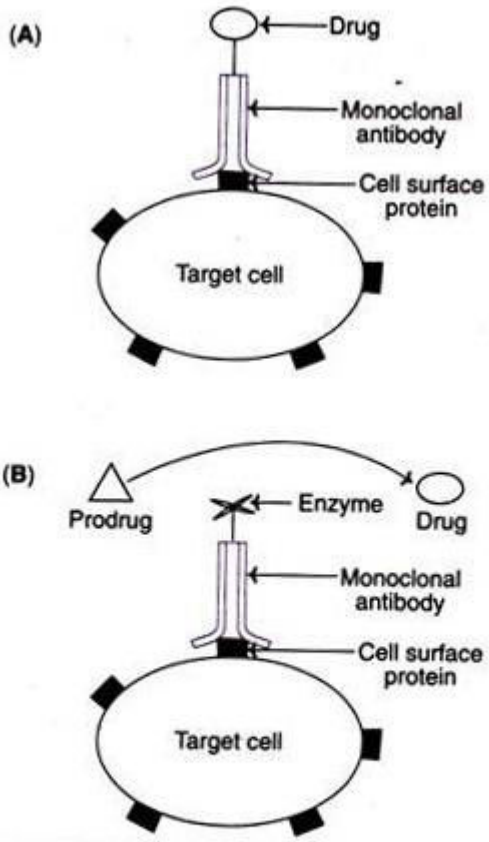

Fig. 17.9 : Monoclonal antibody based drug delivery to the target cells. (A) The drug is bound to MAb (B) The enzyme that converts prodrug to drug is bound to MAb.

In the treatment of certain diseases, a pro-drug (an inactive form of the drug) can be used. This can be enzymatically converted to active drug in the target tissues. For this purpose, the enzyme (that converts pro-drug to drug) is coupled with MAb that is directed against a specific cell surface antigen (Fig. 17.9A). This approach, referred to as antibody-directed enzyme pro-drug therapy (ADEPT), allows an effective delivery of the drug to the cells where it is required.

The following are some examples of enzymes that have been used in ADEPT:

i. Alkaline phosphatase for the conversion of phosphate pro-drugs.

ii. Carboxy peptidase for converting inactive carboxyl pro-drugs to active drugs.

iii. Lactamase for hydrolyzing β-lactam ring containing antibiotics.

MAbs in the dissolution of blood clots:

A great majority of natural deaths are due to a blockage in cornary or cerebral artery by a blood clot (thrombus). Fibrin is the major constituent of blood clot which gets dissolved by plasmin. Plasmin in turn is formed by the activation of plasminogen by plasminogen activator. The blockage of arteries occurs due to inadequate dissolution of blood clots. Tissue plasminogen activator (tPA) can be used as a therapeutic agent to remove the blood clots.

A monoclonal antibody directed against fibrin can be coupled to tPA and used for degradation of blood clots. MAb-tPA complex due to a high affinity gets attached to fibrin (Fig. 17.10). Due to the concentration of tPA at the target spots, there is more efficient conversion of plasminogen to plasmin which in turn dissolves blood clot (fibrin). Good success of clot lysis has been reported by using MAb-tPA complex in experimental animals.

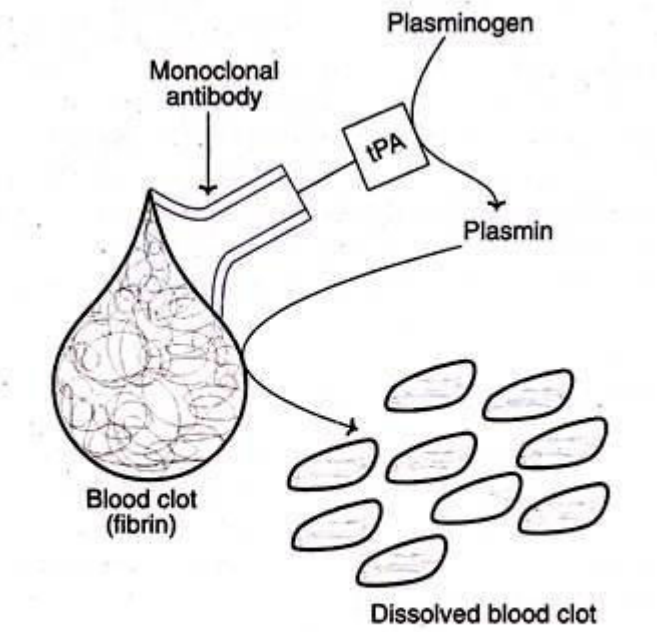

Fig. 17.10 : Monoclonal antibody in the dissolution of blood clot (tPA-Tissue plasminogen activator).

Drug delivery through liposomes coupled to tissue-specific MAbs:

Liposomes are sacs or vesicles formed spontaneously when certain lipid molecules are exposed to aqueous environment. Drug entrapped in liposomes that are coated with MAbs directed against tissue-specific antigens are being tried for drug delivery. Unfortunately, the progress in this approach has been limited, since such liposomes do not reach the target cells. They are retained mostly in the liver and spleen (reticuloendothelial cells), and degraded.

MAbs in radio immunotherapy (RAIT):

The radioisotopes can be coupled to MAbs that are directed against tumor cells. This allows the concentration of radioactivity at the desired sites and a very efficient killing of target cells (tumor cells). The advantage with radio immunotherapy is that conjugated complex need not penetrate the cells, as is required in immunotoxin therapy.

The limitation is that the neighbouring normal cells may also get damaged or killed. This can be minimized by using radioisotopes with short half-lives. Yttrium-90 with a half-life of 64 hours is a suitable isotope to be employed in RAIT. Due to shortage in the supply of yttrium-90, indium-111 is more commonly used.

3. Protein Purification:

Monoclonal antibodies can be produced for any protein. And the so produced MAb can be conveniently used for the purification of the protein against which it was raised. MAbs columns can be prepared by coupling them to cyanogen bromide activated Sepharose (chromatographic matrix). The immobilized MAbs in this manner are very useful for the purification of proteins by immunoaffinity method.

Advantages:

There are certain advantages of using MAbs for protein purification. These include the specificity of the MAb to bind to the desired protein, very efficient elution from the chromatographic column and high degree of purification. Immunoaffinity chromatography is routinely used for the purification of recombinant interferon's. The efficiency of this technique will be obvious from the fact that by a single step, it is possible to achieve more than 5,000 fold purification of interferon-α2.

Disadvantages:

It is not possible to achieve 100% purity of the target protein by immunoaffinity. This is due to the fact that a small quantity of MAb leaks into the elution. Further, MAbs cannot distinguish between the intact target protein and a fragment of it with the antigenic site.

4. Miscellaneous Applications:

(A) Catalytic MAbs (ABZYMES):

Catalysis is the domain of enzymes. The most important common character between enzymes and antibodies is that both are proteins. Further, the binding of an antibody to its antigen is comparable to the binding of an enzyme to its substrate. In both instances, the binding is specific with high affinity and involves weak and non-covalent interactions (electrostatic, hydrogen and van der Waals

forces). The striking difference is that the enzyme alters the substrate (to a product) while the antigen bound to antibody remains unaltered.

Certain similarities between enzyme-substrate interaction and antibody-antigen interaction have tempted researchers to explore the possibility of using antibodies in catalysis. The antibody enzymes, appropriately regarded as abzymes, are the catalytic antibodies. There is a difference in the antibody recognition of an antigen and enzyme recognition of a substrate. While the antibodies recognize in ground state, the enzymes recognize in a transition state (associated with a conformational change of protein).

In fact, it is in the transition condition the catalysis occurs. If a molecule resembling the transition state and conformation (between substrate and product) could be used as a hapten the antibodies so produced should bring about catalysis. This is what precisely is done to create abzymes.

Researchers have produced a hapten-carrier complex which resembles the transition state of an ester undergoing hydrolysis. This hapten conjugate is used to generate anti-hapten monoclonal antibodies. These MAbs could bring about hydrolysis of esters with great degree of specificity (to the transition state to which MAbs were raised).

Besides ester hydrolysis, there are several other types of reactions wherein antibodies can be used. These include hydrolysis of amides and carbonates, cyclization reactions, elimination reactions and bio-molecular chemical reactions. Certain enzymes require cofactors for their catalytic function. MAbs incorporating metal ions have been developed to carry out catalysis.

Lerner and his associates carried out pioneering work in the development of abzymes. They could create a large number of immunoglobulin-gene libraries for the production of antibodies that could be screened for their catalytic function. Abzymes represent a major biotechnological advancement that will have a wide range of applications (cutting of peptides and DNAs, dissolution of blood clots, killing of viruses etc.)

Advantages of abzymes:

The number of naturally occurring enzymes and their catalytic functions are limited. Antibodies, on the other hand, are unlimited, and may be developed to possess recognized site structures, appropriate for the catalytic functions. This makes abzymes versatile with wide ranging catalytic applications. Thus, the area of abzyme technology is very promising, although the studies are at preliminary stages.

Limitations of abzymes:

Despite the progress made in the production and utility of abzymes, it is doubtful whether they will ever match the natural enzymes in their catalytic function. However, the abzymes will be certainly useful for a variety of reactions where the natural enzymes do not have the desired specificities.

(B) Autoantibody Fingerprinting:

The occurrence of autoantibodies and their involvement in certain diseases is well known (e.g. rheumatic arthritis). A new category of individual specific (IS) autoantibodies have been discovered in recent years. These IS-autoantibodies are produced after birth and reach maximum in number by 2 years, and then remain constant for the later part of life.

RFLP -APPLICATIONS IN LIVESTOCK ANIMALS

Recent developments in DNA technologies have made it possible to uncover a large number of genetic polymorphisms at the DNA sequence level, and to use them as markers for evaluation of the genetic basis for the observed phenotypic variability. The markers revealing variations at DNA level are referred to as the molecular markers. Based on techniques used for detection, these markers are classified into two major categories: Hybridization-based markers; and PCR-based markers.

The molecular markers possess unique genetic properties and methodological advantages that make them more useful and amenable for genetic analysis compared to other genetic markers. The possible applications of molecular markers in livestock improvement have been reviewed with reference to conventional and transgenic breeding strategies.

In conventional breeding strategies, molecular markers have several short-range or immediate applications (viz. parentage determination, genetic distance estimation, determination of twin zygosity and freemartinism, and sexing of pre-implantation embryos and identification of disease carrier) and long-range applications (viz. gene mapping, and marker-assisted selection). In transgenic breeding, molecular markers can be used as reference points for identification, isolation and manipulation of the relevant genes, and for identification of the animals carrying the transgenes. The progress in development of molecular markers suggest their potential use for genetic improvement in livestock species.

The progress in recombinant DNA technology and gene cloning during the last two decades has brought in revolutionary changes in the field of basic as well as of applied genetics by providing several new approaches for genome analysis with greater genetic resolution. It is now possible to uncover a large number of genetic polymorphisms at the DNA sequence level, and to use them as markers for evaluation of the genetic basis for the observed phenotypic variability. Though theoretically DNA sequencing is the direct approach to reveal such DNA polymorphism, it has two practical limitations: (i) sequencing needs initial cloning of the gene or DNA fragment at which allelic variation of interest exists, and (ii) it requires suitable and cost-effective method for scoring DNA sequence variation. However, the indirect approach for uncovering of genetic variation at the DNA level using molecular or DNA markers obviates the above limitations.

Since the first demonstration of DNA-level polymorphism, known as the restriction fragment length polymorphism (RFLP), an almost unlimited number of molecular markers have accumulated. Currently, more powerful and less laborious techniques to uncover new types of DNA markers are steadily being introduced. The introduction of polymerase chain reaction (PCR) in conjunction with the constantly increasing DNA sequence data also represents a milestone in this endeavour. The present review is a brief account of molecular markers, and their various applications in livestock improvement.

Molecular markers and their different types

A marker is usually considered as a constituent that determines the function of a construction (Webster's Dictionary). Genetic marker can be defined as any stable and inherited variation that can be measured or detected by a suitable method, and can be used subsequently to detect the presence of a specific genotype or phenotype other than itself, which otherwise is nonmeasurable or very difficult to detect. Such variations occurring at different levels, i.e. at the morphological, chromosomal, biochemical or DNA level can serve as the genetic markers. The markers revealing variations at the DNA level are referred to as the molecular markers, and on the basis of techniques used for their detection, these have been classified into two major categories: Hybridization-based markers, and PCR-based markers.

The hybridization-based markers

These include the traditional RFLP analysis as well, wherein appropriately labeled probes for the genes of importance (e.g. cDNA or genomic DNA sequences) are hybridized on to filter membranes containing restriction enzyme (RE)-digested DNA, separated by gel electrophoresis and subsequently transferred onto these filters by Southern blotting. The polymorphisms are then visualized as hybridization bands. The individuals carrying different allelic variants for a locus will show different banding patterns. Hybridization can also be carried out with the probes (e.g. genomic or synthetic oligonucleotide) for the different families of hypervariable repetitive DNA sequences namely, minisatellite, simple repeats, variable number of tandem repeats (VNTR), and microsatellite[8] to reveal highly polymorphic DNA fingerprinting patterns (DFP).

The PCR-based markers

These have, however, removed the necessity of probe-hybridization step, and have led to the discovery of several useful and easy-to-screen methods. Depending on the type of primers (i.e. primers of specific sequences targeted to a particular region of a genome or primers of arbitrary sequences) used for PCR, these markers can be further sub-divided into the following two groups:

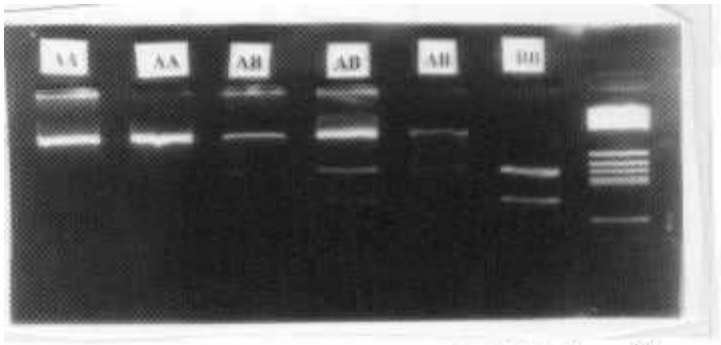

Figure 1. PCR-RFLP of bovine kappa-casein (CSN3) locus. Using sequence-specific primer pair (K1 and K2) a 379 bp fragment (encompassing exon IV and a part of intron V) of CSN3 gene containing a polymorphic *Hin*dIII site was amplified. The amplified fragment was digested with *Hin*dIII, electrophoresed in 3% Nusieve agarose gel and visualized under UV light after staining with ethidium bromide. When the *Hin*dIII site is present, as in case of CSN3 B allele, the amplified fragment is cleaved into two fragments of 156 and 223 bp. In case of CSN3 A, the amplicon remained undigested indicating the absence of *Hin*dIII site. Genotypes of the sample are shown above the lanes. (Source: ref. 83)

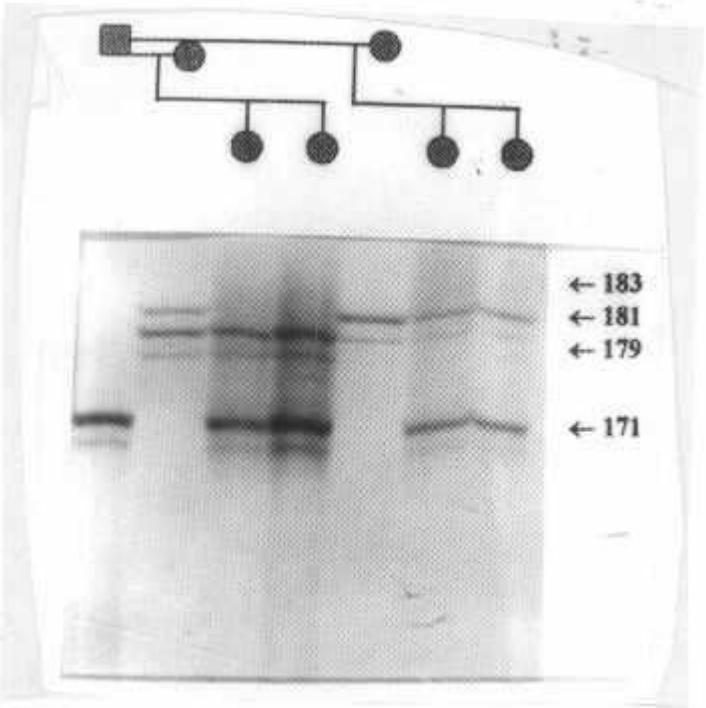

Figure 2. Autoradiograph of gel showing di-nucleotide repeat microsatellite polymorphism in goat. Genomic DNA samples were amplified using bovine BM7160 primer pair. α-^{33}P dCTP was added in reaction mixture. Amplified products were resolved by denaturing acrylamide gel (7%) electrophoresis and visualized by autoradiography. (Source: ref. 84)

(i) The sequence-targeted PCR assays: In this assay system a particular fragment of interest is amplified using a pair of sequence-specific primers. In this category, PCR-RFLP or cleaved amplified polymorphic sequence (CAPS) analysis is a useful technique for screening of sequence variations that give rise to the polymorphic RE sites (Figure 1). Such analysis involves amplification of a specific region of DNA encompassing the polymorphic RE site, and digestion of the amplified DNA fragment with the respective RE. However, for the screening of the sequence variations that do not lead to creation or abolition of restriction sites, other approaches namely allele specific PCR (AS-PCR), PCR amplification of specific alleles (PASA), allele specific oligonucleotide (ASO) hybridization assay, amplification refractory mutation system (ARMS), and oligonucleotide ligation assay (OLA) are used. These assays are based on the principle of high specificity of PCR to selectively amplify specific alleles using primers that match the nucleotide sequence of one, but mismatch the sequence of other allele. The sequence-targeted PCR approach is also employed to reveal simple sequence length polymorphism (SSLP), using a pair of primers that flank the simple sequence repeat (SSR) motifs (Figure 2). If cloned and sequenced microsatellite loci can be subjected to PCR amplification and such microsatellite loci can be recovered by PCR, such loci are termed as sequence tagged microsatellite site (STMS)markers. Microsatellite markers in STMS format can be completely described as information in databases that can serve as common reference points and will allow the incorporation of any type of physical mapping data into the evolving map

(ii) The arbitrary PCR assays: In this assay system, however unlike the standard PCR protocol, randomly designed single primer is used to amplify a set of anonymous polymorphic DNA fragments. It is based on the principle that when the primer is short (usually 8 to 10 mer), there is a high probability that priming may take place at several sites in the genome that are located within amplifiable distance and are in inverted orientation. Polymorphism detected using this method is called randomly amplified polymorphic DNA (RAPD). Based on this principle, several techniques, which do not require any prior sequence knowledge, have been developed. However, they differ in number and length of primers used, stringency of PCR conditions, and the method of fragment separation and detection. In arbitrary primed PCR (AP-PCR), slightly longer primer is used (e.g. universal M13 primer) and amplification products are detected by radiactive or nonradioactive method following polyacrylamide gel electrophoresis. In DNA amplification

fingerprinting (DAF) analysis, shorter primer is used (5 to 8 mer) which reveals relatively greater number of amplification fragments by polyacrylamide gel electrophoresis and silver staining. All these techniques having similar features can be described by a common term multiple arbitrary amplicon profiling (MAAP). Besides these, a number of modifications of the basic MAAP assays (namely, template endonuclease cleavage MAAP and RAPD-RFLP) have been developed as well.

In addition to arbitrary primers, semi-arbitrary primers designed on the basis of RE sites or sequences that are interspersed in the genome such as repetitive sequence elements (*Alu* repeats or SINEs), microsatellites and transposable elements are also used. In the amplified fragment length polymorphism (AFLP) assay, template DNA is digested with two REs, and the resulting restriction fragments are then ligated with adapters and, subsequently, PCR amplification is carried out using specially designed primers which comprise (i) a unique part corresponding to selective bases; and (ii) a common part corresponding to the adapters and the RE site. Microsatellite-primed PCR (MAP-PCR) assay is carried out using microsatellite as the primer.

Applications of molecular markers

Polymorphisms observed at the DNA sequence level have been playing a major role in human genetics for gene mapping, pre- and post-natal diagnosis of genetic diseases, and anthropological and molecular evolution studies. Similar approach for exploitation of DNA polymorphism as genetic markers in the field of animal genetics and breeding has opened many vistas in livestock improvement programmes. Consequently, enormous interest has been generated in determining genetic variability at the DNA sequence level of different livestock species, and in their assessment whether these variations can be exploited efficiently in conventional as well as in transgenic breeding strategies

Gene Therapy

Gene therapy is an experimental treatment that involves introducing genetic material into a person's cells to fight or prevent disease. Researchers are studying gene therapy for a number of diseases, such as severe combined immuno-deficiencies, hemophilia, Parkinson's disease, cancer and even HIV, through a number of different approaches (see video: 'Gene Therapy a new tool to cure human diseases'). A gene can be delivered to a cell using a carrier known as a "vector." The

most common types of vectors used in gene therapy are viruses. The viruses used in gene therapy are altered to make them safe, although some risks still exist with gene therapy. The technology is still in its infancy, but it has been used with some success.

Basic Process of Gene Therapy

Several approaches to gene therapy are being tested, including:

- Replacing a mutated gene that causes disease with a healthy copy of the gene
- Inactivating, or "knocking out," a mutated gene that is functioning improperly
- Introducing a new gene into the body to help fight a disease

In general, a gene cannot be directly inserted into a person's cell. It must be delivered to the cell using a carrier, or vector. Vector systems can be divided into:

	Viral Vectors
	Non-viral Vectors

Currently, the most common type of vectors are viruses that have been genetically altered to carry normal human DNA (see also Wiley database on vectors used in gene therapy trials). Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have tried to harness this ability by manipulating the viral genome to remove disease-causing genes and insert therapeutic ones (see also video 2).

Target cells such as the patient's liver or lung cells are infected with the vector. The vector then unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state.

Types of Gene Therapy

Virtually all cells in the human body contain genes, making them potential targets for gene therapy. However, these cells can be divided into two major categories: somatic cells (most cells of the

body) or cells of the germline (eggs or sperm). In theory it is possible to transform either somatic cells or germ cells.

Gene therapy using germ line cells results in permanent changes that are passed down to subsequent generations. If done early in embryologic development, such as during preimplantation diagnosis and in vitro fertilization, the gene transfer could also occur in all cells of the developing embryo. The appeal of germ line gene therapy is its potential for offering a permanent therapeutic effect for all who inherit the target gene. Successful germ line therapies introduce the possibility of eliminating some diseases from a particular family, and ultimately from the population, forever. However, this also raises controversy. Some people view this type of therapy as unnatural, and liken it to "playing God." Others have concerns about the technical aspects. They worry that the genetic change propagated by germ line gene therapy may actually be deleterious and harmful, with the potential for unforeseen negative effects on future generations.

Somatic cells are nonreproductive. Somatic cell therapy is viewed as a more conservative, safer approach because it affects only the targeted cells in the patient, and is not passed on to future generations. In other words, the therapeutic effect ends with the individual who receives the therapy. However, this type of therapy presents unique problems of its own. Often the effects of somatic cell therapy are short-lived. Because the cells of most tissues ultimately die and are replaced by new cells, repeated treatments over the course of the individual's life span are required to maintain the therapeutic effect. Transporting the gene to the target cells or tissue is also problematic. Regardless of these difficulties, however, somatic cell gene therapy is appropriate and acceptable for many disorders, including cystic fibrosis, muscular dystrophy, cancer, and certain infectious diseases. Clinicians can even perform this therapy in utero, potentially correcting or treating a life-threatening disorder that may significantly impair a baby's health or development if not treated before birth.

In summary, the distinction is that the results of any somatic gene therapy are restricted to the actual patient and are not passed on to his or her children. All gene therapy to date on humans has been directed at somatic cells, whereas germline engineering in humans remains controversial and prohibited in for instance the European Union.

Somatic gene therapy can be broadly split into two categories:

	ex vivo, which means exterior (where cells are modified outside the body and then transplanted back in again). In some gene therapy clinical trials, cells from the patient's blood or bone marrow are removed and grown in the laboratory. The cells are exposed to the virus that is carrying the desired gene. The virus enters the cells and inserts the desired gene into the cells' DNA. The cells grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene therapy is called ex vivo because the cells are treated outside the body.
	in vivo, which means interior (where genes are changed in cells still in the body). This form of gene therapy is called in vivo, because the gene is transferred to cells inside the patient's body.

Gene Doping

Gene therapy for restoring muscle lost to age or disease is poised to enter the clinic, but athletes are eyeing it to enhance performance. The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to improve athletic performance is defined as Gene Doping by the World Anti-Doping Agency (WADA). A complex ethical and philosophical issue is what defines gene doping, especially in the context of bioethical debates about human enhancement. Gene doping could involve the recreational use of gene therapies intended to treat muscle-wasting disorders. Many of these chemicals may be indistinguishable from their natural counterparts. In such cases, nothing unusual would enter the bloodstream so officials would detect nothing in a blood or urine test. For example, gene doping could be used to provide athletes a source of erythropoietin (EPO), a hormone that promotes the formation of red blood cells that is already widely abused in sports. Another candidate gene is Insulin-like Growth Factor 1 (IGF-1) which partly controls the building and repair of muscles by stimulating the proliferation of satellite cells. See also Gene Doping article by Prof. H. Lee Sweeney.

The historical development of policy associated with gene doping began in 2001 when the International Olympic Committee (IOC) Medical Commission met to discuss the implications of gene therapy for sport. It was shortly followed by the WADA, which met in 2002 to discuss genetic enhancement. In 2003, WADA decided to include a prohibition of gene doping within their World Anti-Doping Code, which is formalized in its 2004 World Anti-Doping Code. In 2004, the Netherlands Centre for Doping Affairs (NeCeDo) and the WADA have organized a "Gene Doping" workshop. In addition, NeCeDo has published a report on gene doping as an inventory of the possible applications and risks of genetic manipulation in sports. Although there have been no documented cases of gene doping, the science of gene therapy and interest in the techniques by the sports community has risen to a level that makes gene doping inevitable. The World Anti-Doping Agency (WADA) has already asked scientists to help find ways to prevent gene therapy from becoming the newest means of doping. In December 2005, the World Anti-Doping Agency hosted its second landmark meeting on gene doping, which took place in Stockholm. At this meeting, the delegates drafted a declaration on gene doping which, for the first time, included a strong discouragement of the use of genetic testing for performance. Recently, German scientists from Tübingen and Mainz have developed a blood test that can reliably detect gene doping even after 56 days: "For the first time, a direct method is now available that uses conventional blood samples to detect doping via gene transfer". See news item: Gene Doping Detectable With a Simple Blood Test. Analogous to gene doping, non-therapeutic applications of gene therapy can be envisaged in animals for the purpose of growth stimulation and improved meat production (see also Belgian Blue Bull), for example by growth hormone, myostatin and anabolic hormones. Gene doping to improve sport performance is not limited to humans, but has also interest in for example the sport of horse racing.

APPLICATIONS :FUTURE OF TISSUE ENGINEERING :

Patients suffering from diseased and injured organs are often treated with transplanted organs, and this treatment has been in use for over 50 years. In 1955, the kidney became the first entire organ to be replaced in a human, when Murray transplanted this organ between identical twins. Several years later, Murray performed an allogeneic kidney transplant from a non-

genetically identical patient into another. This transplant, which overcame the immunologic barrier, marked a new era in medicine and opened the door for use of transplantation as a means of therapy for different organ systems.

Modern medicine increases the human lifespan, the aging population grows, and the need for donor organs grows with it, because aging organs are generally more prone to failure. However, there is now a critical shortage of donor organs, and many patients in need of organs will die while waiting for transplants. In addition, even if an organ becomes available, rejection of organs is still a major problem in transplant patients despite improvements in the methods used for immunosuppression following the transplant procedure. Even if rejection does not occur, the need for lifelong use of immunosuppressive medications leads to a number of complications in these patients.

These problems have led physicians and scientists to look to new fields for alternatives to organ transplantation. In the 1960s, a natural evolution occurred in which researchers began to combine new devices and materials sciences with cell biology, and a new field that is now

termed *tissue engineering* was born. As more scientists from different fields came together with the common goal of tissue replacement, the field of tissue engineering became more formally established. Tissue engineering is now defined as "an interdisciplinary field which applies the principles of engineering and life sciences towards the development of biological substitutes that aim to maintain, restore or improve tissue function."Then, after the discovery of human stem cells by Thomson's group in the early 1980s,the field of stem cell biology took shape and suggested that it may one day be possible to obtain and use donor stem cells in tissue engineering strategies, or perhaps even reactivate endogenous stem cells and use them to regenerate failing organs in adult patients.

The fields of stem cells, cell transplantation, and tissue engineering all have one unifying concept-the regeneration of living tissues and organs. Thus, in 1999, William Haseltine, then the Scientific Founder and Chief Executive Officer of Human Genome Sciences, coined the term *regenerative medicine*.

THE BASIC COMPONENTS OF REGENERATIVE MEDICINE STRATEGIES

The field of regenerative medicine encompasses various areas of technology, such as tissue engineering, stem cells, and cloning. Tissue engineering, one of the major areas of regenerative medicine, follows the principles of cell transplantation, materials science, and engineering toward the development of biological substitutes that can restore and maintain normal function. Tissue engineering strategies generally fall into two categories: the use of acellular scaffolds, which depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth, and the use of scaffolds seeded with cells. Acellular scaffolds are usually prepared by manufacturing artificial scaffolds or by removing cellular components from tissues via mechanical and chemical manipulation to produce acellular, collagen-rich matrices. These matrices tend to slowly degrade on implantation and are generally replaced by the extracellular matrix (ECM) proteins that are secreted by the in-growing cells. Cells can also be used for therapy via injection, either with carriers such as hydrogels or alone.

1. Biomaterials for use in regenerative medicine

In the past, synthetic materials were introduced to replace or to rebuild diseased tissues or parts in the human body. The manufacture of new materials, such as tetrafluoroethylene (Teflon) and silicone, opened a new field of research that led to the development of a wide array of devices that could be applied for human use. Although these devices could provide structural support or replacement, the functional component of the original tissue was not restored. However, studies in cell biology, molecular biology, and biochemistry allowed a better understanding of the ECM and its interaction with cells in the tissues of the body, as well as interactions with growth factors and their ligands, and as a result, new biomaterials were designed with these interactions in mind.

In tissue engineering, biomaterials replicate the biological and mechanical function of the native ECM found in tissues in the body. Biomaterials provide a three-dimensional space in which cells can attach, grow, and form new tissues with appropriate structure and function. They also allow for the delivery of cells and appropriate bioactive factors (e.g., cell adhesion peptides, growth factors) to desired sites in the body. Because most mammalian cell types are anchorage-dependent and will die if no cell-adhesion substrate is available, biomaterials provide this substrate while allowing delivery of cells with high loading efficiency. Biomaterials can also provide mechanical support against *in vivo* forces so that the predefined three-dimensional structure of a tissue-engineered organ is maintained during tissue development.

The ideal biomaterial should be biodegradable and bioresorbable to support the replacement of normal tissue without inducing inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection or necrosis. Because biomaterials provide temporary mechanical support while the cells undergo spatial reorganization into tissue, a properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, while in

late development, it should have begun degradation such that it does not hinder further tissue growth.The degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate to ensure that the concentration of these degradation products in the tissues remains at a tolerable level.

Generally, three classes of biomaterials have been utilized for engineering tissues: naturally derived materials (e.g., collagen and alginate), acellular tissue matrices (e.g., bladder submucosa and small intestinal submucosa),and synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), and poly (lactic-co-glycolic acid) (PLGA).These classes of biomaterials have been tested with respect to their biocompatibility.Naturally derived materials and acellular tissue matrices have the potential advantage of biological recognition. However, synthetic polymers can be produced reproducibly on a large scale with controlled properties such as strength, degradation rate, and microstructure.

2. Cells for use in cell therapy and tissue engineering

1) Native cells

When native cells are used for tissue engineering, a small piece of donor tissue is dissociated into individual cells. These cells are expanded in culture and either injected directly back into the host or attached to a support matrix and then reimplanted. The source of donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous. The preferred cells to use are autologous cells, where a biopsy of tissue is obtained from the host, the cells are dissociated and expanded in culture, and the expanded cells are implanted into the same host.The use of autologous cells, although it may cause an inflammatory response, avoids rejection, and thus the deleterious side effects of immunosuppressive medications can be avoided.

Ideally, both structural and functional tissue replacement will occur with minimal complications when autologous native cells are used. However, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement has been the inherent difficulty of growing specific cell types in large quantities. Even when some organs, such as the

liver, have a high regenerative capacity *in vivo*, cell growth and expansion *in vitro* may be difficult. By studying the privileged sites for committed precursor cells in specific organs, as well as exploring the conditions that promote differentiation, one may be able to overcome the obstacles that limit cell expansion *in vitro*. For example, urothelial cells could be grown in the laboratory setting in the past, but only with limited expansion. Several protocols were developed over the past two decades that identified the undifferentiated cells and kept them undifferentiated during their growth phase. With the use of these methods of cell culture, it is now possible to expand a urothelial strain from a single specimen that initially covered a surface area of 1 cm^2 to one covering a surface area of 4,202 m^2 (the equivalent of one football field) within 8 weeks. These studies indicated that it should be possible to collect autologous bladder cells from human patients, expand them in culture, and return them to the donor in sufficient quantities for reconstructive purposes. Major advances have been achieved within the past decade on the possible expansion of a variety of primary human cells, with specific techniques that make the use of autologous cells for clinical application possible.

Most current strategies for tissue engineering depend on a sample of autologous cells from the diseased organ of the host. However, for many patients with extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous human cells cannot be expanded from a particular organ, such as the pancreas. In these situations, stem cells are envisioned as being an alternative source of cells from which the desired tissue can be derived. Stem cells can be derived from discarded human embryos (human embryonic stem cells), from fetal tissue, or from adult sources (bone marrow, fat, skin).

3. Stem cells for use in tissue engineering

1) Embryonic stem cells

Human embryonic stem (hES) cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated but pluripotent state (self-renewal), and the ability to differentiate into many specialized cell types. They can be isolated by aspirating the inner cell mass from the embryo during the blastocyst stage (5 days post-fertilization) and are usually grown on feeder layers consisting of mouse embryonic fibroblasts or human feeder cells. More recent reports

have shown that these cells can be grown without the use of a feeder layer and thus avoid the exposure of these human cells to mouse viruses and proteins. These cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages when grown by use of current published protocols. In addition, hES cells are able to differentiate into cells from all three embryonic germ layers *in vitro*. Skin and neurons have been formed, indicating ectodermal differentiation. Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation. Pancreatic cells have been formed, indicating endodermal differentiation. In addition, as further evidence of their pluripotency, embryonic stem cells can form embryoid bodies, which are cell aggregations that contain all three embryonic germ layers while in culture and can form teratomas *in vivo*. However, there are many ethical and religious concerns associated with hES cells because embryos are destroyed in order to obtain them. Thus, the use of these cells is currently banned in many countries.

2) Stem cells from somatic cell nuclear transfer

Stem cells for tissue engineering could also be generated through cloning procedures. There has been tremendous interest in the field of nuclear cloning since the birth of the cloned sheep Dolly in 1997, but actually, Dolly was not the first animal produced by using nuclear transfer. In fact, frogs were the first successfully cloned vertebrates derived from nuclear transfer. However, in the frog experiment, the nuclei used for cloning were derived from non-adult sources. In fact, live lambs were produced in 1996 by using nuclear transfer as well, but they were produced from differentiated epithelial cells derived from embryonic discs. The significance of Dolly was that she was the first mammal to be derived from an adult somatic cell by use of nuclear transfer. Since then, animals from several species have been grown by using nuclear transfer technology, including cattle, goats, mice, and pigs.

Two types of nuclear cloning, reproductive cloning and therapeutic cloning, have been described, and a better understanding of the differences between the two types may help to alleviate some of the controversy that surrounds these technologies. Banned in most countries for human applications, reproductive cloning is used to generate an embryo that has the identical genetic material as its cell source. This embryo can then be implanted into the uterus of a female

to give rise to an infant that is a clone of the donor. On the other hand, therapeutic cloning is used to generate early stage embryos that are explanted in culture to produce embryonic stem cell lines whose genetic material is identical to that of its source. These autologous stem cells have the potential to become almost any type of cell in the adult body, and thus would be useful in tissue and organ replacement applications. Therefore, therapeutic cloning, which has also been called somatic cell nuclear transfer, may provide an alternative source of transplantable cells. According to data from the Centers for Disease Control and Prevention, an estimated 3,000 Americans die every day of diseases that could have been treated with stem cell-derived tissues.With current allogeneic tissue transplantation protocols, rejection is a frequent complication because of immunologic incompatibility, and immunosuppressive drugs are usually required. The use of transplantable tissue and organs derived from therapeutic cloning could lead to the avoidance of immune responses that typically are associated with transplantation of non-autologous tissues.

While promising, somatic cell nuclear transfer technology has certain limitations that require further study before this technique can be applied widely in tissue or organ replacement therapy. First, the efficiency of the cloning process is very low, as evidenced by the fact that most embryos derived from the cloning process do not survive. To improve cloning efficiency, further improvements are required in many of the complex steps of nuclear transfer, such as the enucleation process for oocytes, the actual transfer of a nucleus to this enucleated oocyte, and the activation process that instructs the cloned oocytes to begin dividing. In addition, cell cycle synchronization between donor cells and recipient oocytes must be accomplished.

3) Reprogramming and generation of iPS cells

4) Within the past few years, exciting reports of the successful transformation of adult somatic cells into pluripotent stem cells through genetic "reprogramming" have been published. Reprogramming is a technique that involves de-differentiation of adult somatic cells (such as fibroblasts) to produce patient-specific pluripotent stem cells. This process is especially exciting

because it allows pluripotent stem cells to be obtained without the use of embryos. Also, cells generated by reprogramming are genetically identical to the somatic cells used (and thus to the patient who donated these cells) and should not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an "induced pluripotent state (iPS)." They examined 24 genes that were thought to be important for embryonic stem cells and identified 4 key genes that, when introduced into the reporter fibroblasts via retroviral vectors, resulted in drug-resistant cells. These were *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*. The resultant iPS cells possessed the immortal growth characteristics of self-renewing embryonic stem cells, expressed genes specific for embryonic stem cells, and generated embryoid bodies *in vitro* and teratomas *in vivo*. When iPS cells were injected into mouse blastocysts, they contributed to a variety of cell types. However, although iPS cells selected in this way were pluripotent, they were not identical to embryonic stem cells. Unlike embryonic stem cells, chimeras made from iPS cells did not result in full-term pregnancies. Gene expression profiles of the iPS cells showed that they possessed a distinct gene expression signature that was different from that of embryonic stem cells. In addition, the epigenetic state of the iPS cells was somewhere between that found in somatic cells and that found in embryonic stem cells, suggesting that the reprogramming was incomplete.

These results were improved significantly by Wernig and Jaenisch in July 2007. Fibroblasts were infected with retroviral vectors and selected for the activation of endogenous *Oct4* or *Nanog* genes. Results from this study showed that DNA methylation, gene expression profiles, and the chromatin state of the reprogrammed cells were similar to those of embryonic stem cells. Teratomas induced by these cells contained differentiated cell types representing all three embryonic germ layers. Most importantly, the reprogrammed cells from this experiment could form viable chimeras and contribute to the germline-like embryonic stem cells, suggesting that these iPS cells were completely reprogrammed. Wernig et al observed that the number of reprogrammed colonies increased when drug selection was initiated later (day 20 rather than day 3 post-transduction). This suggests that reprogramming is a slow and gradual process and may explain why previous attempts resulted in incomplete reprogramming.

It has recently been shown that reprogramming of human cells is possible. Yamanaka generated human iPS cells that are similar to hES cells in terms of morphology, proliferation, gene expression, surface markers, and teratoma formation. Thompson's group showed that retroviral transduction of the stem cell markers *OCT4*, *SOX2*,*NANOG*, and *LIN28* could generate pluripotent stem cells. However, in both studies, the human iPS cells were similar but not identical to hES cells. Although reprogramming is an exciting phenomenon, our limited understanding of the mechanism underlying it currently limits the clinical applicability of the technique, but the future potential of reprogramming is quite exciting.

5) Amniotic fluid and placental stem cells

An alternate source of stem cells is the amniotic fluid and placenta. Amniotic fluid and the placenta are known to contain multiple partially differentiated cell types derived from the developing fetus. We isolated stem cell populations from these sources, called amniotic fluid and placental stem cells (AFPSC), that express embryonic and adult stem cell markers. The undifferentiated stem cells expand extensively without feeders and double every 36 hours. Unlike hES cells, the AFPSC do not form tumors *in vivo*. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking can be induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal, and hepatic lineages. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue. Examples of differentiated cells derived from AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-proteingated inwardly rectifying potassium (GIRK) channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue engineered bone. The cells could be obtained either from amniocentesis or chorionic villous sampling in the developing fetus, or from the placenta at the time of birth. The cells could be preserved for self-use and used without

rejection, or they could be banked. A bank of 100,000 specimens could potentially supply 99% of the US population with a perfect genetic match for transplantation. Such a bank may be easier to create than with other cell sources, because there are approximately 4.5 million births per year in the USA.

6) Adult stem cells

Adult stem cells, especially hematopoietic stem cells, are the best understood cell type in stem cell biology. The presence of stem cells in the adult was first discerned by Till and McCulloch, who were investigating the mechanisms by which the bone marrow could regenerate after exposure to radiation. However, adult stem cell research remains an area of intense study, because their potential for therapy may be applicable to a myriad of degenerative disorders. Within the past decade, adult stem cell populations have been found in many adult tissues other than the bone marrow and the gastrointestinal tract, including the brain, skin, and muscle. Many other types of adult stem cells have been identified in organs all over the body and are thought to serve as the primary repair entities for their corresponding organs. The discovery of such tissue-specific progenitors has opened up new avenues for research.

A notable exception to the tissue-specificity of adult stem cells is the mesenchymal stem cell (MSC), also known as the multipotent adult progenitor cell. This cell type is derived from bone marrow stroma. Such cells can differentiate *in vitro* into numerous tissue types and can also differentiate developmentally if injected into a blastocyst. Multipotent adult progenitor cells can develop into a variety of tissues including neuronal, adipose, muscle, liver, -lungs, spleen, and gut tissue but notably not bone marrow or gonads.

In addition, stem cells derived from adipose tissue may also be an autologous and self-renewing cell source. Adipose-derived stem cells (ADSCs) have been shown to differentiate into a variety of cell phenotypes, and since they are easily obtained, they show great promise for future types of reconstructive surgery based on tissue engineering and there have been several clinical trials using these cells. Wilson and Mizuno have both provided excellent, detailed reviews of these.

Research into more differentiated types of adult stem cells has, however, progressed slowly, mainly because investigators have had great difficulty in maintaining adult non-mesenchymal stem cells in culture. Some cells, such as those of the liver, pancreas, and nerve, have very low proliferative capacity *in vitro*, and the functionality of some cell types is reduced after the cells are cultivated. Isolation of cells has also been problematic, because stem cells are present in extremely low numbers in adult tissue. While the clinical utility of adult stem cells is currently limited, great potential exists for future use of such cells in tissue-specific regenerative therapies. The advantage of adult stem cells is that they can be used in autologous therapies, thus avoiding any complications associated with immune rejection.

Applications in Environment and Agriculture:

Genetically engineered poultry, swine, goats, cattle, and other livestock also are beginning to be used as generators of pharmaceutical and other products, potential sources for replacement organs for humans, and models for human disease. The technology to produce foreign proteins in milk by expressing novel genes in the mammary glands of livestock already has advanced beyond the experimental stage, with some of the products currently in clinical trials (Colman, 1996; Murray and Maga, 1999). In theory, transgenic animals can provide milk that is more nutritious for the consumer, or that is enhanced for certain protein components that might be valuable for manufacturing cheese or other dairy products. However, the largest investments in the technology to date have been made by pharmaceutical companies interested in producing enzymes, clotting factors, and other bioactive proteins in milk.

Companies also are interested in farm animals as possible sources of replacement organs for humans. Transplantation is an accepted and successful treatment for organ failure, but there is an enormous shortage of available human organs. As there are ethical and practical concerns related to the use of donor organs from primates, the pig, in particular, is being considered as an alternative. Unfortunately, humans express antibodies to a carbohydrate epitope (terminal 1,3-galactose residues) that is present on the surface of pig cells (Sandrin et al., 1993). As a result, the xenograft immediately becomes a target for acute rejection. To remedy this situation, pigs will be produced that lack the 1,3 galactosyl transferase enzyme (Tearle et al., 1996; Dai et al., 2002; Lai et al., 2002).

Although the mouse, because of its small size, short generation times, fecundity, and well-studied genetics has become the animal of choice for providing models for human disease, farm species might provide alternatives where the mouse is inappropriate. One possible future scenario is the creation of specific gene knockouts in farm animals in order to mimic human disease in a large animal model. For example, McCreath et al. (2000), have generated genetically-engineered sheep carrying a mutated collagen gene, and have suggested that such animals could serve as models for the human connective tissue disease *osteogenesis imperfecta*.

The development of such technologies and others yet to be conceived and their incorporation into agricultural and biomedical practice raises concerns about whether the end products can be consumed safely, whether there are likely to be unwanted effects on the environment, and whether animal welfare will be adversely affected. The goal of this report is to identify concerns that will aid the federal regulatory agencies in evaluating the possibility of such adverse outcomes. However, before proceeding further, it is perhaps helpful to understand what is meant by biotechnology and to appreciate how far such biotechnology already has been incorporated into current agricultural and biomedical practice. It also is clear that the concerns of the public are focused on some of the more recent technologic advances relating to gene transfer between organisms that would not normally interbreed and to assisted reproductive procedures, such as somatic nuclear cell transfer to create so-called clones (Eyestone and Campbell, 1999; Box 1.1). Many of these recent advances have not yet left the experimental stage, but it is clear that several, including transgenic finfish, which are soon likely to be commercialized, are likely to assume importance both in agriculture and medicine.

A Definition of Cloning

The verb “to clone” and the noun “clone” have a range of meanings and interpretations. The noun is derived from the Greek word klōn, meaning a twig. Its original use in English was to describe asexually produced progeny, and it has been in familiar use in horticulture for centuries. “To clone” in this context, therefore, means to make a copy of an individual. “Clone” was later adopted into the parlance of modern cellular and molecular biology to describe groups of identical cells, and replicas of DNA and other molecules. Monozygotic twins are clones, but the term has recently become popularized in the media to mean an individual, usually a fictitious human, grown from a single somatic cell of its parent. The first reports of animal cloning were in the late 1980s and were the result of the transfer to anucleated oocytes of nuclei from blastomeres (cells from early, and

presumably undifferentiated, cleavage-stage embryos), a technique that is referred to as blastomere nuclear transfer or BNT, in this report. Cloning of sheep, cattle, goats, pigs, mice, and, more recently, rabbits and cats, by transplanting a nucleus from a somatic, and presumably differentiated, cell into an oocyte—from which its own genetic material has first been removed—was achieved about a decade later (Wilmut et al., 1997; reviewed by Westhusin et al., 2001), leading to the speculation that humans also could be cloned. It is important to note that somatic cell nuclear transfer (SNT) also can be used to produce embryonic stem cells, giving researchers the opportunity to obtain undifferentiated stem cells that are genetically matched to the recipient for research and therapy, which is independent of the discussion here regarding the use of SNT for reproductive cloning of animals. Neither BNT nor SNT result in an exact replica of an individual animal, although the progeny are very similar to each other and to their donor cell parent. Any genetic dissimilarity is likely due to the cytoplasmic inheritance of mitochondria from the donor egg, which possesses its own DNA, and to other cytoplasmic factors, which seem to have the potential to influence the subsequent "reprogramming" of the transferred somatic cell genome in such a way that spatial and temporal patterns of gene expression in the embryo are affected as it develops (Cummins, 2001; Jaenisch and Wilmut, 2001). For these reasons, many scientists have objected to the use of the term clone in the context of somatic cell nuclear transfer. The committee acknowledges this shade of meaning and has attempted to make the appropriate distinction when the term *clone* is used. Nevertheless, *clone* is now so widely accepted as a synonym for somatic cell nuclear transfer—not just by the public at large—but also by embryologists and other biologists, that the committee has retained it rather than attempt to replace it with a more precise, but cumbersome, phrase.

THE ORIGINS OF BIOTECHNOLOGY IN ANIMAL AGRICULTURE

Biotechnology literally is technology based on biology; it is the application of scientific and engineering principles to the processing or production of materials by biologic agents to provide goods and services. The application of biotechnology to animals has a long history, beginning in Southwest Asia after the last ice age, when humans first began to trap wild animal species and to breed them in captivity, initially for meat and fiber and later for transport and milk. Of the approximately 48,000 mammalian species, fewer than 20 have been successfully domesticated (Diamond, 1999). Other than cats and dogs, only five of these species (cattle of the *Bos* genus, whose ancient ancestor is the now extinct auroch; sheep derived from the Asiatic mouflon species; goats, which are descended from the benzoar goat of West Asia; pigs derived from captured wild

boars; and horses, which originated from now extinct wild horses that roamed the steppes of Southern Russia) are found worldwide (Diamond, 1999; Box 1.2). As pointed out by Hale (1969) and Diamond (1999), the animals that have been successfully domesticated and farmed share and exhibit a unique combination of characteristics. They are relatively docile, are flexible in their dietary habits, and can grow and reach maturity quickly on a herbivorous diet, and breed readily in captivity. They also have hierarchical social structures that permit humans to establish dominance over them, and are adapted to living in large groups. They do not include species that generally have a tendency to be fearful of humans or disturbed by sudden changes in the environment. Our ancestors no doubt based their selection methods for improving their herds and flocks on how easy the animals were to farm, as well as on potential agricultural value. In turn, the animals are adapted to thrive in a domesticated environment.

The fact that the modern breeds of these species differ so markedly from their progenitor species is a reflection of how quickly directed breeding can act. The modern Holstein, which dominates the contemporary United States dairy industry, little resembles its ancestors of only a half-century ago. Milk production per cow increased almost threefold between 1945 and 1995 (Majeskie, 1996), largely as a result of breeding from select bulls. There has been an accompanying drop in the number of cows, land devoted to dairy production and in manure produced. On the downside, the cows have a tendency towards lameness, are considerably less fertile than in the 1940s, and are frequently maintained in a herd for no more than 2–3 years or 2–3 lactations (Pryce et al., 2000; Royal et al., 2000), and represent a very narrow genetic lineage (Weigel, 2001). The export of these animals and their lineages to Europe and elsewhere is assuring the globalization of both the benefits and drawbacks of the American Holstein. Analogous changes are ongoing in the swine industry, where the pressure to produce lean, fast-growing animals of uniform size is leading to the abandonment of old breeds (Notter, 1999). Paradoxically, unless the old livestock breeds are eaten, sheared or milked, they will not survive.

The dog (*Canis familiaris*), on the other hand, provides an interesting example of the range of phenotypes that can be derived by selection within a single species. Dogs are believed to have originated in several separate domestications from wolves (*Canis lupus* and *Canis rufus*) and coyotes (*Canis latrans*) before the domestication of livestock. They have undergone remarkable modifications in size and behavior over short periods of intense selection and to provide the diversity observed in modern breeds. This reflects the enormous pool of genetic variation within the species (Wayne and Ostrander, 1999), but (possibly) also the fixation of new mutations into different genetic lineages. Inbreeding of dog breeds, as of domestic livestock, has led to a major narrowing of intrabreed variability (Zajc et al., 1997).

The same kinds of selective pressures that molded the large farm animal species has led to the creation of the modern breeds of farmed fowl, which include chickens, ducks, geese, and turkeys domesticated for their meat, eggs, and feathers. As in the dairy industry, there has been a remarkable improvement in the productivity of the poultry industry over the last 60 years. Between 1940 and 1994, yearly egg production per laying hen increased from 134 to 254, mainly as a result genetic selection. The broiler industry has shown similar

gains (Pisenti et al., 1999). In 1950, a commercial bird took 84 days to reach a market weight of 1.8 kilogram. By 1988, this market weight was reached by only 43 days (Pisenti et al., 1999) on about half the amount of feed (Lacy, 2000).

Scientific breeding, combined with better nutrition and veterinary care, clearly has produced breeds of animals that are remarkably productive, although sometimes strikingly different in habits and appearance from those farmed early in the twentieth century. The practice has also led to a loss of many breeds of livestock and fowl, and a decline in genetic diversity within the breeds that survive. For example, it has been estimated that there were several hundred specialty lines of chicken in North America at the beginning of the last century, whereas the number of commercial hybrid strains now available through suppliers is fewer than 10 (North and Bell, 1990).

Aquatic animals, including finfish and shellfish, now are farmed, and specific breeds that have been selected for growth and other traits are established now in the largest industrial sectors of aquaculture, such as channel catfish, rainbow trout, and Atlantic salmon. The growth and quality of such animals are also amenable to genetic engineering through modern biotechnology. Genetically engineered or highly selected aquatic species present special problems in terms of confinement, as the features that might make them attractive commercially might pose risks to the genetic base of their wild relatives with which they can interbreed (Hallerman and Kapuscinski, 1992b).

Insects also have been domesticated for farming. The two best-known examples are the honeybee and silkworm; considerable genetic gains in productivity have provided strains of these insects far removed from the ancestral species from which they were derived. Attempts to develop strains of honeybee with improved resistance to pathogens and silkworms that produce proteins other than silk are on the horizon. Insects, like fish, are especially difficult to confine so that “escapes” are almost inevitable. In addition, insects, including ones that can be engineered transgenically, are likely to continue to be used as part of biocontrol programs for pest insects and invasive plant species and, as such, might be intentionally released into the environment. There will almost certainly be attempts to replace or to infiltrate native populations with insects that have been engineered in such a manner that they are less of a pest or unable to transmit pathogens (Hoy, 2000). Private-sector companies already have begun to farm recombinant proteins (antibodies,

cytokines, enzymes, and bioactive peptides) from insect larvae. Whereas the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) regulates the release of insects for pest management, it is unclear which agency is responsible for protecting against accidental release of insects from mass rearing factories. Horizontal gene transfer, disruption of ecosystems, and native species extinctions are among the potential hazards that arise from permanent releases of transgenic arthropods into the environment (Hoy, 2000).

The traditional kind of biotechnology emphasized at the beginning of this section relies upon natural breeding procedures to select valuable phenotypes from the variation in the existing gene pool of a species and is beyond the purview of this report, even though it has contributed so successfully to modern-day production agriculture. It is firmly entrenched in our agricultural communities, and many are generally conversant with its benefits and risks. Importantly, other forms of research-driven biotechnologies, based on improved insight into reproductive physiology and endocrinology, embryology, genetics, and animal health also have made their way into standard farming practices over the last 75 years (Box 1.2). A few of the procedures listed extend the boundaries of biotechnology to the development of organisms that have a combination of traits generally not attainable in nature through conventional breeding and are not themselves without controversy. Some of those listed are perceived by both scientists and lay people as endangering human health or as adversely affecting animal welfare or the environment. Certain of the technologies even can have unintended, long-term consequences on the economics of agriculture itself. Finally, some of the concerns raised about the technologies in Box 1.2 are quite relevant to those listed in Box 1-3. Although several of these technologies remain experimental and have not yet become a part of standard agricultural practice, others (e.g., commercialization of transgenic fish) are undergoing government review for commercial approval. It is these newer technologies on which this report is focused. For these reasons, it is worth while discussing Box 1.2 and some of the issues that these technologies have raised before moving on to the ones associated with Box 1.3.

CONCERNS REGARDING EXTANT TECHNOLOGIES

Animal Health

There are well-established guidelines for the application of technologies that maintain animal health, such as standard vaccination against viral and bacterial diseases. Indeed considerable efforts are being made to expand the

range of such technologies in order to prevent epidemic spread of disease in flocks and herds, which are particularly at risk when farmed under intense conditions (BBC, 2001). Even the

therapeutic use of antibiotics to treat animals that have bacterial infections or are in danger of becoming infected seems not in itself to be controversial, except when antibiotics of medical importance to humans are employed.

Subtherapeutic Use of Antibiotics

The U.S. Food and Drug Administration (FDA) approved antibiotics as feed additives for farm animals in 1951. Their use since has been extended to fish farming, particularly with the global spread and dramatic increase of aquaculture in tanks and pond-like structures where antibiotics are used for prevention and control of disease rather than to enhance growth (NRC, 1999). The treated animals are found to grow more quickly and utilize feed more efficiently than animals on regular feed. At least 19 million pounds of antibiotics are used annually for subtherapeutic purposes in animal agriculture, and generally are added to feed and water (NRC, 1999). Some of these compounds, used on livestock, including penicillin, tetracycline, and fluoroquinolone used on livestock, also are prescribed to treat human illnesses, and the practice has been shown in a few instances to contribute to antibiotic resistance of human pathogens (Chiu et al., 2002; Molbak et al., 1999). It now is generally accepted in the scientific and medical communities that antibiotic resistance can be exacerbated by the widespread improper use of antibiotics. What remains controversial is whether agriculture contributes sufficiently to the problems associated with resistant pathogens to justify a complete curtailment of their use as growth promoters (DANMAP, 2000; Stephenson, 2002). A recent report from the National Research Council (NRC, 1999) failed to find a definitive link between the agricultural use of antibiotics in animal feed drinking water and antibiotic resistance of human pathogens. The report states, “The use of drugs in the food production industry is not without some problems and concerns, but does not appear to constitute an immediate public health concern.” Since that report was released, additional information, raising further concerns, has been released (Fey, 2000; Gorbach, 2001). Consequently, the practice remains under intense scrutiny and is opposed by some scientific and medical organizations.

Assisted Reproductive Procedures

Artificial insemination (AI), and the later, associated use of frozen semen, sire testing and sire selection are all part of a combinatorial approach to improve the genetic quality of farmed species.

AI, when first introduced into agriculture,
elicited an enormous outcry from farmers, the press, and religious groups. It was claimed to be against the laws of God, a repugnant practice that would lead to abnormal outcomes, and

economically unsound (Herman, 1981; Foote, 1996). It gradually has become an accepted practice in agriculture, as well as in human and veterinary medicine. The ability to freeze semen and maintain a high degree of fertilizing ability after thawing extended the power of AI, since a few select bulls could be utilized to inseminate many females in different geographic areas. Such bulls could be tested, not only for fertility, but also for their ability to sire progeny that produced copious amounts of milk. By maintaining accurate records, breeding value estimations of particular bulls could be calculated. The result was the remarkable increase in milk production, noted earlier. On the other hand, the process is leading to potentially destructive inbreeding since many of the select bulls are related. Inbreeding coefficients among modern Holsteins and Jersey breeds are now about 5 percent and rising (Weigel, 2001). The outcome might be inbreeding depression and broad susceptibility to the epidemic spread of disease. There also has been a remarkable recent loss of fertility, with successful pregnancies resulting from first insemination dropping from more than 40 percent to as low as 20 percent or less in some herds as milk yields have risen (Pryce et al., 2000; Royal et al., 2000).

Embryo recovery and transfer provides the opportunity for a particularly valuable animal to parent many more offspring in her lifetime than would be otherwise possible (Seidel, 1984). The embryos also can be frozen and then either stored or transported before they are used to initiate a pregnancy. It is a relatively common technology and has been used to produce an estimated 40,000 to 50,000 thousand beef calves every year (NAAB, 1996). The approach is to induce, by using hormones, the maturation and release of more than a single egg from the ovaries (superovulation; Driancourt, 2001). Then, the animal usually is inseminated with semen from an equally select bull, and the embryos are collected and transferred individually, or in pairs, to the reproductive tract of less valuable cows, which carry the calf to term. Modern technologies also provide the possibility of freezing the embryos and determining their gender prior to transfer. The main concern with this technique, as with the AI-associated technologies discussed above, is that it can lead to narrowing of the genetic base of the breed, in this case involving both parents. A related technique is to use a needle to aspirate immature oocytes from the ovaries (in the case of livestock the oocytes often are taken from slaughtered animals at an abattoir) and to mature the oocytes for about one day in a culture containing hormones. At the stage when the oocytes reach a point midway through the second division of meiosis, they are fertilized with live sperm. In rare instances, fertilization is achieved by a single sperm or sperm head, which is injected through the tough outer zona pellucida of the oocyte, either beneath the zona or directly into the cytoplasm (intracytoplasmic injection, or ICSI). Whatever method is used for fertilization, the resulting zygotes usually are then cultured

until the embryo reaches a more advanced stage of development. In humans, of course, these combined techniques form the basis of *in vitro* fertilization procedures and have resulted in hundreds of thousands of normal infants, but the techniques also have become an important means of producing embryos for experimental purposes in agricultural research. Importantly, *in vitro* maturation of oocytes underpins cloning and transgenic technologies where large numbers of competent, matured oocytes are needed to provide the many eggs necessary for nuclear transfer and pronuclear injection, respectively .*In vitro* fertilization also is used commercially to preserve the genome of particularly valuable animals that have infertility problems such as blocked oviducts or that respond poorly to superovulation a technique described below. This commercial application of IVF is a relatively uncommon, with about 4,000 calves born from its use annually. Few concerns have been raised about this technique, which essentially is identical to that employed for *in vitro* fertilization in humans, although some animal welfare issues have been raised.In order to manage breeding programs more intensively, control over the reproductive cycles of livestock by hormonal intervention has increased. In general the technologies are relatively benign and involve injecting the animal with hormones, usually to stop progression through the existing estrous cycle and sometimes to mimic the events that lead to selection of one or more mature follicle(s) that will ovulate. Superovulation is a technique designed to mature a cohort of follicles simultaneously, with result that several eggs are ovulated simultaneously. A hormone treatment analogous to that used to produce a timed ovulation in the large farm animals is used to induce gonadal maturation in fish. None of these techniques have raised public health concerns, since the hormones are similar or identical to those in normal reproduction and the amounts used within the physiologic range.

Splitting or bisecting embryos became an esoteric but well-established practice in the 1980s in order to provide zygotic twins. The pieces of the embryo—usually "halves," which are genetically identical in terms of both their nuclear and mitochondrial genes are placed in an empty zona (the protective coat around early embryos) before being transferred to different recipient mothers to carry them to term. It is estimated that only a very small number of the calves are produced in this manner. Nevertheless, these animals have been introduced into commercial herds, and have produced progeny; their milk and meat are consumed by the public.

Cloning by nuclear transplantation from embryonic blastomeres is an expensive procedure that also has its origins in the 1970s . What distinguishes it from somatic cell nuclear transfer, the technology that led to the creation of Dolly and much of the controversy over human cloning, is the stage of development at which the nuclei are transferred (Wilmut et al., 1998). In the older procedure, the cells or blastomeres used were from the so-called morula stage of cell development (although some were from the cleavage stage and others from the blastocyst stage) when the

embryo still is an undifferentiated mass and its cells presumed still capable of forming all tissues of a fetus.

The cloning technologies of embryos splitting (EMS) and embryonic nuclear transfer (NT) were introduced into dairy cattle breeding in the 1980s. The Animal Improvement Programs Laboratory of the USDA's Agricultural Research Service (ARS) is responsible for tracking the performance of dairy cattle throughout the U.S. Recently, working with the Holstein Association, they evaluated the performance of cloned Holsteins produced by EMS and NT (H.D. Norman, USDA–ARS, personal communication). The numbers of EMS and NT clones were documented by gender and birth year. All NTs were from embryos rather than adult cells. Through 2001, there were a total of 2,226 EMS (754 males and 1,472 females) and 187 NT (61 males and 126 females) Holstein clones registered. Of female EMS clones, 921 had yield records, and 551 had noncloned full siblings with yield records. Of the 126 female NT clones, 74 had yield records, but only 11 had noncloned full siblings. These familial relationships were used to compare the performance of cloned and noncloned full siblings for standardized traits and genetic evaluations as part of the national evaluation program. These standardized traits included total milk yield, fat content (by weight and pecent), protein content (by weight and percent), somatic cell score, and productive life (in months). Also calculated were yield from contemporaries and predicted transmitting ability. Norman and his colleagues concluded that the numbers of clones have decreased for EMS males and for all NT clones over the past decade. Animals that were selected for cloning were slightly superior genetically to the contemporary population mean for yield traits; the yields of NT clones were similar to, and those of EMS clones were slightly less than, those of their noncloned full siblings.

"Modern" cloning involves taking an unfertilized egg, removing its chromosomes, and introducing the nucleus from a differentiated cell of the animal to be cloned, which is frequently an adult . The introduced nucleus is reprogrammed by the cytoplasm of the egg and directs the development of a new embryo, which is then transferred to a recipient mother to allow it to develop to term. The offspring formed will be identical to their siblings and to the original donor animal in terms of their nuclear DNA, but will differ in their mitochondrial genes and possibly also in the manner their nuclear genes are expressed or biochemically engineered Cloning from blastomeres, the older of the two procedures, has been reported to result occasionally in large calves (and lambs), the so-called large offspring syndrome.

Hormone-Treated Cattle

Among the most contentious technologies used in animal agriculture is the use of steroid hormones to increase the rate of weight gain and to reduce accumulation of fat deposits of young heifers and steers as part of the "finishing" process prior to slaughter . The steroids are administered by slow release from a plastic implant embedded beneath the skin of the ear, which provides "physiologic" circulating levels of the hormone in the bloodstream. The hormones used are mainly Zeranol (in Ralgro™), a naturally occurring fungal metabolite (zearalenone) with estrogenic action; estradiol, progesterone, and testosterone, or mixtures of these steroids (in various Synovex™ formulations); and trenbolone . Concern about these hormones is probably, in part, a legacy of diethylstilbestrol, which was eventually banned from use in the poultry and beef industry because of its adverse effects on humans. However, the amounts of present-use compounds consumed from meat derived from treated cattle are small, and numerous scientific studies generally have indicated that these residues exist at such low concentrations that they pose little risk to consumers, provided good veterinary practices are employed (e.g., using the correct number of implants and placing implants correctly in the ear cartilage), although the U.S. Geological Survey has recently documented the presence of hormones in a number of streams and rivers . Despite the scientific evidence for safety, the European Union implemented a ban on U.S. beef imports, valued at over $100 million per year in 1989 . A concern that has not been extensively examined so far is whether these hormones pose any sort of environmental threat through their leaching into soil and water. For example, two recent studies have shown that a commonly used androgenic growth promotor—trenbolone—has been found in groundwater near cattle feedlots, and that this growth promotor has androgenic effects.

Bovine Somatotropin

The use of bovine somatotropin (BST) to increase milk yield from dairy cows has had a similar checkered history and is the subject of trade disputes. Currently banned in Europe even for experimental studies, BST was approved by the FDA for use in U.S. dairy cattle in 1993 because testing had revealed no concerns regarding consumer safety . The Monsanto product, Posilac™, now is widely used throughout the U.S. dairy industry, where milk production can be increased as much as 30 percent in well managed, appropriately fed herds, without adversely affecting the quality or composition of the milk. The BST, which is almost indistinguishable in sequence from the natural hormone, is present in low concentrations in milk, but has no biologic activity in humans. The level of IGF-1, the hormone induced by BST, is somewhat elevated but within the "physiologic range" for cows and is probably digested along with other milk proteins in the adult stomach, although it might have biologic activity in the intestine of neonates . In its assessment, the

FDA did not report that BST or IGF-1 pose any risk either in humans or animals that consume cows' milk. As with other technologies that increase productivity, a concern frequently raised is why more milk is needed when the developed world appears to have more than enough of the product. One answer is that increased productivity translates into fewer animals, producing less waste and utilizing less land—an extremely important consideration for future land management use. The greatest concerns about BST are probably in the area of animal welfare. High-yield milking cows show a greater incidence of mastitis than lower-producing cows, but studies have shown that mastitis is not exacerbated by BST administration . Another concern—a practical one for the dairy industry—is a recent trend to breed heifers only once and then to sustain milk production for as long as 600 days by using BST. Lengthening lactation via BST in second calf and older cows is a larger contributor to having fewer calves per lifetime in the herd than first-calf heifers. The result has been a shortage of replacement heifers for producers, since only one calf is born during the milking life of the animal .

Marker-Assisted Selection

Marker-assisted selection involves establishing the linkage between the inheritance of a particular trait—which might be desirable, as in the case of milk yield—or undesirable, as in susceptibility to a disease, with the segregation of particular genetic markers. Thus, even if the gene that controls the trait is unknown, its presence can be inferred from the presence of the marker that segregates with it. This technology, which is particularly important for studying
complex traits governed by many genes, has only recently become a factor in animal breeding and selection strategies). Its use likely will increase exponentially as the industry incorporates the data from the various genome sequencing projects and as the density of useful, segregating markers increases on the chromosomes of the species. Initially, animals will be screened for genes that control simple traits, such as horns, which are undesirable in cattle, and halothane sensitivity, which segregates with metabolic stress syndrome in pigs. With time, easily identifiable markers will be chosen that accompany the many genes controlling more complex traits such as meat tenderness and taste, growth, calf size, and disease resistance. The approach has enormous potential for improving the quality of agricultural products, disease resistance, and other traits but could be misused . For example, stringent selection of prime animals could potentially narrow genetic diversity even more than is evident at present. Use of the technique also could maximize short-term gain in productivity but at the expense of longer-term improvement due to what has been termed polygenic drag . In essence, the cumulative effect of genes with effects too small to be exploited in

a marker-assisted selection program could contribute more to increasing desired traits than genes with major effects. However, marker-assisted selection might be a powerful measure to counter inbreeding by providing genetic measures of heterozygosity, encouraging breeding strategies that maintain diversity at the majority of sites in the genome, and allowing the genetic potential of rare breeds and wild ancestors to be utilized and incorporated into mainstream agriculture.

Chromosome Set Manipulation in Mollusks and Finfish

Altering the chromosome complement of an animal can be a useful way of rendering that animal infertile, and is exploited widely in the production of fish and mollusks. Well-timed application of high or low temperatures, certain chemicals, or high hydrostatic pressure to newly-fertilized groups of eggs can interfere with extrusion of the second polar body (the last step in meiosis), resulting in "triploid" individuals with three, instead of the usual two, chromosome sets (e.g., for oysters; Allen et al., 1989). A later treatment can suppress the first cell division of the zygote, resulting in "tetraploid" individuals with four sets of chromosomes. Crossing tetraploids, which are fertile in some species, with normal diploids can then produce large numbers of triploids (Scarpa et al., 1994). Such chromosome set manipulations have been applied to cultured marine mollusks to produce confined stocks of triploids that are unable to reproduce. This application is of particular importance, as some of the shellfishes most suited to aquaculture are not indigenous to a given area and can pose ecologic risks to native species should they or their larvae escape confinement and enter natural ecosystems (USDA, 1995). Induction of triploidy reduces the likelihood that an introduced species would establish self-sustaining populations, because such animals are theoretically sterile. For example, the triploid Suminoe oyster (*Crassostrea ariakensis*) is being assessed for oyster production in the Chesapeake Bay, where diseases complicate restoration of the native Eastern oyster (*C. virginica*). Should triploidy prove an effective means for reproductive confinement, culture of sterile Suminoe oysters could support the recovery of the declining Chesapeake oyster production industry.

Another benefit of producing sterile mollusks is in maintaining product quality throughout the year. The meat quality of oysters is high just before they spawn, but low after spawning. The product quality of reproductively sterile, triploid oysters remains high year-round. Hence, triploid stocks of Pacific oyster (*Crassostrea gigas*) provide a tangible benefit to aquaculturists, and now make up almost half of commercial production in the Pacific Northwest.

Unfortunately, repeatable induction of 100 percent triploidy on a commercial scale poses a considerable technical challenge. Non-triploid larvae within batches of larvae easily can go

undetected if their frequency is low (USDA, 1995). Should triploidy be desired for purposes of maintaining product quality and the species is indigenous to an area, no harm is posed. If, on the other hand, triploidy is to be utilized for reproductive confinement purposes, the presence of reproductively fertile individuals—even in low numbers—might establish progeny and a self-sustaining population. There also are indications that a small percentage of triploid oysters can progress to a "mosaic" state, with diploid cells arising within the background of triploid cells, leading to the possibility that they could produce viable gametes (Calvo et al., 2001; Zhou, 2002).

Triploidy often has been used to reduce the likelihood that introduced finfish species would establish self-sustaining populations. Use of all-female triploid stocks has been suggested as a means of achieving reproductive confinement of transgenic fishes, including Atlantic salmon (the leading candidate for commercialization). As with mollusks, however, repeatable induction of 100 percent triploidy poses a considerable technical challenge, and commercial net pen operations produce hundreds of thousands of salmon, with many escaping.

Another technology used on finfish is to farm monosex fish stocks (Beardmore et al., 2001), which are preferred by producers either because one gender grows faster or larger than the other (e.g., males in catfish and tilapia, females in rainbow trout), or because certain species (e.g., tilapia) attain sexual maturity before reaching harvest size. Monosex populations have been established in several ways, but most reliably through hormone-induced gender reversal. All-male fry can be produced by direct administration of testosterone in feed, or all-females by administration of estrogens. Monosex stocks also can be produced indirectly by gender reversal and progeny testing to identify XX males for producing all-female stocks, as in trout (Bye and Lincoln, 1986) and salmon (Johnstone and Youngson, 1984), or YY males for producing all-male stocks, as in tilapia .

Harms ,Hazardss and Risks:

The charge of the committee was to identify, but not to quantify, risk issues concerning products of animal biotechnology, and to provide criteria for selection of those risk issues considered most important that need to be addressed or managed for the various product categories. In order to provide criteria for selection of risk issues, it is important to understand how risk is determined. As outlined in Chapter 5 and as set forth by NRC (1983; 1996), a *hazard*: is an act or phenomenon that has the potential to produce harm, and *risk* is the likelihood of harm resulting from exposure to the hazard. This committee used the NRC (1996) definition of risk to develop a set of working steps to prioritize concerns. Because risk is the product of two probabilities: the probability of exposure, and the conditional probability of harm given exposure has occurred, the steps in risk analysis are to: (1) identify the potential harms, (2) identify the potential hazards that might produce those harms, (3) define what exposure means and the likelihood of exposure and 4) quantify the

likelihood of harm given that exposure has occurred. (The committee notes that risk analysis in other fields can and does include additional steps in risk assessment; see Kapuscinski, 2002). Multiplying the resulting probabilities then was used to prioritize risk. While absolute probabilities are difficult to determine at this time, relative rankings from high to low are possible based on available evidence for each category. The risks, harms, and hazards are different for each chapter because the issues are different (i.e., a hazard resulting in an animal wellbeing concern might not be an environmental or human health concern).

Intellectual Property Rights:

Use of biotechnology is not a new concept. Humans have used biotechnology since the dawn of civilization. The practice of animal biotechnology began more than 8000 years ago with the domestication and selective breeding of animals. Invention of genetic code in the mid-1950s pioneered the present concept of animal biotechnology.

Initially, the biotechnology progressed at slow pace and remained a subject of little attention. Development of techniques viz., genetic engineering or recombinant DNA (rDNA) techniques, cell culturing and manipulation, cell fusion, production of monoclonal antibodies, and gene-editing heralded a new era of revolution in biomedical sciences.

Meanwhile, it was realized that above innovations in conjunction with reproduction technologies could modify the cells, embryos, and animals, and indeed, the outcomes were amazing. This initiated the concerns about intellectual property and its protection in biotechnology applied to animals.

At present, the animal biotechnology is a major area of basic and applied biological research aimed to develop veterinary vaccines, molecular diagnostics, transgenic or gene-edited animals for diagnosis of human diseases, using genetically engineered animals for therapeutics and organs. All this involves intellectual inputs, and outcomes are of enormous economic importance.

The term intellectual property (IP) is generic legal term that describes various types of intangible assets and laws which protect the innovations and applications of thoughts, ideas, and information of profitable value. In broader sense, IP protection is about the laws related to patents, copyrights, trademarks, trade secrets, and other similar rights .

Currently, a number of processes and methods applied to biotechnology are under legal protection, and most of the processed initiated in the USA. Later on, other countries also started competing in

new biotechnological markets. It became important to them to amend their national laws in order to protect and boost investment in biotechnology. Despite the fact that international consensus is lacking on how biotechnology has to be treated, the biotechnology innovators opt for patenting their invention.

Webber has insisted that research outcomes of pharmaceutical sector, any biotech start-up or academia should be protected by patents. This is because enormous efforts, hard work, and financial inputs are needed to develop a novel drug or product out of hundreds and thousands of lead compounds. It is therefore prerogative for a company or institute to protect their innovative products from unauthorized duplication.

The patenting grants legal, time-bound monopolies to the eligible scientists or inventors . Similarly, the trade secrets (information related to a formula, compilation, device or program or technique) can be protected by law that varies depending on a country and its legislation. The owner of trade secret is known as originator.

Intellectual Property Laws

Science plays key role in economic prosperity of the nation. Economy is strong when science and technology are allowed to do well. It is the technology that delivers goods and services, hence, directly or indirectly, the science is correlated to generate the revenue.

The very basic purpose of IP protection is to allow the right holder to prohibit others from using the IP (patents, copyrights, trademarks, and trade secrets) rights in well-defined ways.

Activities of practicing scientist inextricably intersect with business world. Some inventions are protected by various types of IP protection. It is important to take into consideration the business legal factors before opting for a particular strategy . Some IPs are protected by simple regulatory norms, others need stringent IP protection.

Types of IPs in Animal Biotechnology

Patenting Genetically Modified Organisms

Patents cover various technologies used to modify mice or rat genome and the stem cells. For instance, the French institute CERBM and IGBMC (Centre Européen de Recherche en Biologie et en Médecine–L'Institut de génétique et de biologie moléculaire et cellulaire) have patents of describing the use of the drug tamoxifen to induce Cre recombinase activity in vivo in a transgenic

mouse for a transgenic mouse model, named "Oncomouse," whose germ and somatic cells carry recombinant activated oncogene . The transgenic mouse was developed at Harvard Medical School (Brown 2000).

By 2004, around 600 animal patents had been granted worldwide, 80% in the USA, and most relating to "animal models" for biomedical investigations. Besides the USA and European Union, only three countries had allowed patents for experimental animals .

IP Issues in In Silico Biology

Bioinformatics and in silico methods are indispensable tools to analyze gigantic "omics" sequence data. The bioinformatics programs are used to predict hypothetical gene, proteins, and metabolic athways from the sequence data of microorganism . In silico methods are used in computational modeling in medicinal computational chemistry, predicting drug-target interactions and developing (Quantitative) Structure-Activity Relationships .

Potentially patentable bioinformatics programmes include lines of code, algorithms, data content, data structure, and user interfaces. Several bioinformatics algorithms are available for analyzing sequence data and predicting genes and proteins from the data. Many of them are freely accessible while others need payments. Some commercial companies provide services to analyze the sequence data. Success or failure of bioinformatics and in silico biology needs the appropriate use of legal tools for protecting and utilizing the intellectual property. Patenting of products discovered using in silico procedures is still a debatable issue and needs a due attention.

Therapeutics and IP

Vaccines, monoclonal antibodies, and antibiotics provide protection against infectious diseases. As there are many forms of vaccines, and components of vaccines, delivery systems, and distribution networks, a variety of IP protections are applicable to vaccines . With advances in understanding molecular biology of viruses, there is increase in the application of virus sequences and viral gene expression strategies to diagnose and treat the diseases. This extends the scope of patenting multiple constituents of vaccines. Patents have also been granted for recombinant antigen-based rapid sero-diagnosis of viral infectious diseases, synthetic peptide antigens, and multiple antigenic peptide (MAP) assays for detecting viral infectious diseases of livestock.

Outlook and Challenges

Applications of biotechnology and molecular biological tools in animal sciences have reached new horizons. In present scenario, the transgenic animals have a large role in developing pharmaceutical industries and medicinal research. Developed countries have already realized potential of biotechnology-oriented industries and the importance of patenting the genetically modified animals.

Surprisingly, the world's richest biodiversity is present in countries that are poor, and lack resources and necessary wherewithal to transform their bioresources into products and earn the revenue. Also, the deprived or developing countries are unable to invest revenue to promote the research and provide adequate patent protection for genetically modified animals.

The legal costs involved in IP can be minimized by strategic planning and diligence. Due to high-controversial nature and processes involved in legal grant of protection for animal biotechnology, the short-term developments are likely to take place at national and regional levels. We emphasize whether a person works in a biotech start-up or a university or in commercial pharmaceutical company, a sound knowledge of patent system is necessary to protect the outcomes of novel research process or the end product.

IP impinges on almost every invention a scientist does. Scientists and researchers working in the fields of animal biotechnology and genetic engineering, however, need training to deal with complex issues of IP, their rights and obligations.

BIOSAFETY GUIDELINES:

Biosafety guidelines are a set of policies, rules, and procedures necessary to observe by personnel working in various facilities handling microbiological agents such as bacteria, viruses, parasites, fungi, prions, and other related agents and microbiological products. Institutions requiring strict adherence to these biosafety guidelines include clinical and microbiological laboratories, biomedical research facilities, teaching and training laboratories and other healthcare institutions (e.g., clinics, health centers, hospital facilities). These guidelines are intended to provide proper management and regulation of biosafety programs and practices implemented at all levels of the organization.

Essential components of the biosafety guidelines contain some or all the following, depending on the facility: microbiological risk assessment and identification; specific biosafety measures, which cover the code of practice, physical plant such as laboratory design and facilities, equipment acquisition and maintenance, medical surveillance, staff training, safe handling of chemicals, with fire, radiation and electricity safety, among others. Additional components may be included such as commissioning and certification guidelines for the facilities.

Biosafety guidelines must be made clear, practical and suitable for each facility and must be available for easy reference by all staff, must be reviewed, and updated regularly. While it provides guidance in the application of biosafety practices, this technical guide cannot solely ensure a safe working environment without the commitment of each person to adhere adequately to the biosafety guidelines at all times. Continuous research on biosafety can improve the development of future guidelines[1]

History of Biosafety

A significant milestone on biosafety initially referred to as "microbiological safety" dates back to 1908 where Winslow described a new method of examination to count bacteria present in the air[2] A survey reviewed by Meyer and Eddie in 1941 described laboratory-acquired brucellosis which also revealed that similar infections could pose a threat to non-laboratorians.[[3] Later in 1947, the NIH Building 7 had the first peacetime research laboratory especially tailored for microbiological safety. These historical landmarks and breakthroughs are just a few of the more studies which untied the importance and relevance of biosafety in healthcare and research institutions.

The principle and profession of biosafety have developed together with the history of the American Biological Safety Association (ABSA). As briefly described by the Federation of American Scientists, the first meeting was held in 1955 with the members of the military, as the focus addressed "The Role of Safety in the Biological Warfare Effort". Succeeding meetings attendees included the US Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH), universities, laboratories, hospitals and representatives from the industries. From then, written regulations covered the shipment of biological agents, safety training and programs, with the development of biological safety level classification[4] International issues on biosafety and studies on the individual or group of agents became the focus in the 1980s. At present, aside

from studies focusing on specific biohazard level or pathogen, new strategies were developed to enhance risk assessment capacities, biosecurity, and biocontainment measures including the regulation of biosafety through national and international policies. Other industries such as in agriculture and biotechnology are now considering biosafety application.

Epidemiology of Laboratory-Acquired Infections (LAIs)

Laboratory-acquired infections (LAIs) were considered significant because of the high risk in the laboratory workforce relative to the public, although the exposure to infectious agents can be higher in other groups of healthcare workers. Sulkin and Pike in 1949 studied several works of literature and mail surveys with an attempt to evaluate the risk of infection associated with employment in a clinical or research laboratory. Follow-up studies and reviews led to the identification and description of hazards unique to these laboratories, which later formed a basis for the development of approaches to prevent the emergence of LAIs.[[5][6]

The incidence of laboratory-acquired infections varies among institutions conducting surveys to a specific or group of laboratories and facilities. Monitoring and evaluation of LAIs are still absent for many institutions which could be caused by the difficulties in the reporting schemes and lack of accurate data interpretation. For instance, reporting of LAI is not similar to the reporting of notifiable diseases which is highly regulated for each healthcare institution across countries as implemented by their ministries of health. Laboratory-acquired infections may not always manifest as a disease entity. An example would be a person infected with tuberculosis, who could have an infection with TB bacilli but with no signs and symptoms, thus, cannot be considered as TB disease. No national and global recording and reporting of LAI is in place. Though LAI incidence is reported in several publications recently, the variables and the levels of measurement under study differ, hence, combination and comparison of such studies is not a simple task. However, the need for data collection for current LAIs should highlight the importance of improving biosafety which outweighs the above issues. LAI databases were then created to contain all recently published studies and to verify its relevant findings. While these address the need for acquiring new information, it will not replace the reporting schemes implemented by individual institutions.

In 2018, Siengsanan-Lamont and Blacksell presented the results of a rapid review about LAI studies within the Asia-Pacific. Studies from 1982 to 2016 included several agents, some of these include: Shigella flexneri (Australia), Mycobacterium tuberculosis (Japan), Rickettsia typhi (South Korea), SARS-CoV (Singapore, China, Taiwan), Dengue (South Korea, Australia) and Ralstonia picketti (Taiwan) to name a few. Regarding potential risks for zoonotic diseases, viruses predominate, followed by bacteria and parasites. The importance of risk assessment and management was also emphasized, including preventive practices. Strict biosafety measures is a must for these working environments to protect themselves and the community[7]

Specimen Requirements and Procedure

All specimens collected from patients require the application of biosafety measures. It starts with the instructions provided by the healthcare worker to the patient. Clear statements with explanations and step-by-step procedures are necessary, especially for patients who will collect the specimen. Healthcare workers, including laboratory staff, should be well-oriented especially when they are to collect specimens directly from patients. Personal protective equipment (PPE) must be worn at all times during the specimen collection.[8][9] Universal precautions must be applied accordingly.[10]

Several procedures exist for collecting sterile and non-sterile sample specimens. Better strategies were developed recently to minimize hazards either during and after sending the specimens into the laboratory. For example, the use of the evacuated tube system (ETS) prevented the contact of the patient's blood from the site of extraction to the phlebotomist and the external environment during venipuncture.[11] This is much safer than the previous practice of manual transferring blood samples from the syringe to the tube.[12] Sputum collected in a clear and transparent container will aid in efficient visualization and assessment of sputum quality which is safer than reopening the cap.[13] These are examples where applying biosafety measures become crucial in the pre-analytical phase.[14]

Diagnostic Tests

Clinical laboratory scientists (medical technologists) must perform laboratory procedures both accurately and safely.[15] PPE must be worn out while inside the premises of the laboratory and

throughout the diagnostic procedure. There is a proper sequence of donning (putting on) and doffing (removing) PPE as recommended by the US Centers for Disease Prevention and Control (CDC). Generally, donning starts with gowning, wearing of a mask (or respirator), goggles (or face shield) and gloving. Doffing may be done by removing the gloves, goggles, gown, and mask followed by the proper hand washing.

Pathogen-specific and risk-specific biosafety measures are shown to be more practical and cost effective.[4] For example, low and medium-risk procedures do not need a containment facility and infrastructure which are designed only for high-risk procedures. Safe handling and processing of specimens can be conducted in biological safety cabinets (BSCs) to prevent inhalation of generated aerosols when performing a microbiological procedure.[16] The purpose of using BSCs must be well differentiated from using fume hoods, in which the latter is only necessary for handling chemicals and not for infectious microorganisms. When dealing with specimens, keep hands away from the face and should remain inside the cabinet. Unnecessary movements inside the BSC is prohibited to prevent the changes in the flow of air. For instance, the crossing of arms during the laboratory procedure is inadvisable. Also, ensure to disinfect the BSC before use. In procedures done in the absence of a BSC, a well-ventilated area must be secured and maintained before considering it as a bench work area. When gloves become heavily contaminated, wear new gloves. Do not reuse gloves in other procedures nor soiled masks or respirators. Molecular biology laboratories perform procedures which require the use of different rooms for sample preparation, DNA extraction, amplification and sequencing, thus, the need for additional biosafety measures.[17]

Proper disposal of wastes is necessary to prevent disease transmission.[18] Waste segregation must be appropriately employed (e.g., infectious and non-infectious waste). Waste disposal via burning may not be practical nowadays. Hence, alternative disposal mechanisms must be finalized and institutionalized in each healthcare institution.[19] Environmental impact is always a consideration when making decisions for waste disposal. Treatment facilities (i.e., treatment plants) are used to remove contaminants before sewage gets released into the environment. Specific steps should be written on standard operating procedure manuals and work instructions intended for laboratory staff involved.[20]

Recording and reporting procedures must be free from possible contamination and should of in a clean and dedicated space.[21] Similarly, wearing gloves when encoding via computer or when using the phone is forbidden.

Because of the complexity of the laboratory work, one must be well-trained and supervised to perform biosafety measures at work, while non-authorized personnel must have restricted access to the laboratory, especially when a diagnostic test is in process.

Testing Procedures

The development of biosafety guidelines is part of the overall quality management systems implementation. For newly established facilities, ensure biosafety before the start of operations. Workflow inside the laboratory must facilitate an efficient means for carrying out processes by the laboratorian. Activities involving dirty areas (e.g., a specimen receipt, sample preparation, etc.) should be kept separate from the clean areas (e.g., microscopy, use of automated instrumentation, recording of results, etc.). Procedures for laboratory workflow can be tested through observation and evaluation by a designated biosafety officer, laboratory supervisor or an independent consultant who can conduct monitoring activities and provide technical assistance.

For labs using BSCs, a smoke pattern test using in-house or commercial testers may be regularly performed to assess for good airflow before use. Anemometers may be used to check for air velocity. BSC certification provided by a service professional must be secured before use and continually re-certified once a year.[22][23]

Before performing any laboratory test, the provision of required training on biosafety to the laboratory workforce is vital, either as a focused training program or as part of the training curriculum for certain laboratory procedure. Laboratory managers, section heads and supervisors should receive biosafety training as well, including topics on risk management and biosafety program implementation. Effective supportive supervision of laboratory staff working in any facility is a key factor for sustained implementation of quality laboratory services.[24]The integration of the monitoring of biosafety practices with monitoring of laboratory processes should

proceed based on set criteria or standards. Certain indicators which indirectly assess the overall biosafety may include the presence of an updated procedure manual and work instructions, a list of trained staff with regular competency or proficiency tests, with regular quality control and maintenance of laboratory equipment. Regular medical consultation for staff can early detect the risk of infection. Moreover, the presence of laboratory signage such as a biohazard symbol to recommended sites of the facility, with a well-organized mechanism for disposal of wastes can significantly minimize the risk of accidents and incidents both inside and outside the laboratory. Laboratory accreditation and certification may also aid in ensuring that biosafety measures get implemented in accordance with the written guidelines.[25][26]

Interfering Factors

Several factors impede the application of laboratory-acquired biosafety measures within the facility. These may include, but not limited to:

The absence of a technical document containing specific biosafety guidelines

- Poor biosafety skills (for example, on spills management) because of lack of training
- The continuous presence of laboratory hazards and increased risk due to the lack or inadequate of risk assessment and management
- Use of substandard laboratory supplies
- Poor equipment maintenance

Biosafety guidelines are more likely to be poorly implemented in facilities because of:

- Poorly written guidelines, including the adoption of generic, nonspecific procedures
- Unclear roles and responsibilities for each staff involved
- Lack of review and updating process of existing guide
- Poor dissemination and access to such guidelines