CLASS: II M.Sc Biotech **COURSE NAME: Animal Biotechnology**
COURSE CODE: 17BTP302 **BATCH-2017-2019**

Animal Biotechnology

17BTP302 Semester III

Total Hours/Week: L : 4: T: 0 : P: 0 Marks: Internal; 40 : External : 60 Total 100

Scope: Biotechnologists explore and develop new technologies using molecular biology, embryo manipulation, cell and tissue culture to manipulate and explore the genomes of animals for ways to improve the live-stock for food production and biomedical purpose.

Objective: To provide an experience for the students in an interdisciplinary research program connecting animal genomics with animal reproduction and biotechnology**.**

UNIT -I

Animal cells: culture media, types of media, balances salt solutions. Physical, chemical and metabolic functions of different constituents of culture medium; role of carbon dioxide, serum, growth factors, glutamine in cell culture; serum and protein free defined media and their applications.

UNIT -II

Cell culture: Types, disaggregation of tissue, primary culture, established culture; suspension culture, organ culture, three dimensional culture and tissue engineering, feeder layers; cell synchronization; cryopreservation. Biology and characterization of cultured cells, tissue typing; cell – cell interaction; measuring parameters of growth; measurement of cell death – apoptosis and its determination.

UNIT-III

Molecular cell techniques: cell transformation- physical, chemical and biological methods; manipulation of genes; cell and organism cloning; green fluorescent protein and its application. Gene therapy.

UNIT -IV

Embryology: Collection and preservation of embryos; culturing of embryos; gametogenesis and fertilization in animals; types of cleavage pattern; role of maternal contributions in early embryonic development; *In vitro* fertilization and stem cell research.

UNIT -V

Transgenics: Transgenic animals; production and application; transgenic animals as models for human diseases; transgenic animals in live- stock improvement; expression of the bovine growth hormone; transgenics in industry. Ethical issues in animal biotechnology.

References:

T1: Text Book of Animal Biotechnology. 2006. R.Sasidhara. MJP Publishers

R1: Ranga, M. M. (2007). *Animal Biotechnology*. (3rd ed.). Jodhpur: Agrobios.

W1: www. biotechnology4u.com/animal biotechnology

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LECTURE PLAN

S.No	I	Topics to be covered	Support Materials
UNIT I			**8hr**
1.	1	Culture media	W1: 1
2.	1	Types of media	T1: Pg: 40 - 42
3.	1	Balances salt solutions	W1: Pg: 15
4.	1	Physical, chemical constituents of culture medium	W1: Pg: 8.
5.	1	Metabolic functions of different constituents of culture medium	W1: Pg: 6
6.	1	Role of carbon dioxide, serum, growth factors, glutamine in cell culture	W1: Pg: 7 -11
7.	1	Serum and protein free defined media and their applications	W1: Pg: 18 -37
8.	1	Revision	
UNIT II			**11hr**
9.	1	Types, disaggregation of tissue	T1: Pg: 46 - 51
10.	1	Primary culture, established culture; suspension culture,	T1: Pg: 58 - 63
11.	1	Organ culture, three dimensional culture and tissue engineering	W1: 14- 17
12.	1	Feeder layers; cell synchronization;	T1: Pg: 39
13.	1	Cryopreservation	W2: Pg: 87
14.	1	Biology and characterization of cultured cells,	T1: Pg: 64 - 67

15.	1	Tissue typing	W1: Pg: 55
16.	1	Cell – cell interaction;	W1: Pg: 57
17.	1	Measuring parameters of growth of cells	T1: Pg: 73 - 75
18.	1	Measurement of cell death – apoptosis and its determination	W1: Pg: 57 - 59
UNIT III			**10hr**
19.	1	Molecular cell techniques	T1: Pg: 81 - 83
20.	1	Cell transformation	T1: Pg: 84 -85
21.	1	Physical methods of cell transformation	T1: Pg: 86 - 95
22.	1	Chemical methods of cell transformation	T1: Pg: 96 - 103
23.	1	Biological methods of cell transformation	T1: Pg: 113 - 128
24.	1	Manipulation of genes	T1: Pg: 103 - 109
25.	1	Cell and organism cloning	T1: Pg: 103
26.	1	Green fluorescent protein and its application	T1: Pg: 68 - 69
27.	1	Gene therapy	T1: Pg: 232 - 249
28.	1	Revision	
UNIT IV			**10 hr**
29.	1	Collection and preservation of embryos	T1: Pg: 174 - 175
30.	1	Culturing of embryos	T1: Pg: 175 - 176
31.	1	Gametogenesis	T1: Pg: 178 - 179

32.	1	Fertilization in animals	T1: Pg: 179 - 180
33.	1	Types of cleavage pattern	T1: Pg: 181 - 183
34.	1	Role of maternal contributions in early embryonic development	T1: Pg: 184 - 185
35.	1	*In vitro* fertilization	T1: Pg: 171 - 174
36.	1	Stem cell research	T1: Pg: 421 - 437
37.	1	Revision	
38.	1	Unit test	
UNIT V			**9 hr**
39.	1	Transgenic animals; production and application	T1: Pg: 136- 161
40.	1	Transgenic animals as models for human diseases	T1: Pg: 142 - 147
41.	1	Transgenic animals in live- stock improvement	T1: Pg: 136 - 161
42.	1	Expression of the bovine growth hormone	T1: Pg: 161 - 162
43.	1	Transgenics in industry	T1: Pg: 156
44.	1	Ethical issues in animal biotechnology	T1: Pg: 452 - 460
45.	1	Revision	
46.	1	ESE question paper revision	
47.	1	ESE question paper revision	
48.	1	ESE question paper revision	
	Total Hours = (Unit I+ II + III + IV + V) = 48 hr		

UNIT-1

SYLLABUS

Animal cells: culture media, types of media, balances salt solutions. Physical, chemical and metabolic functions of different constituents of culture medium; role of carbon dioxide, serum, growth factors, glutamine in cell culture; serum and protein free defined media and their applications.

Introduction

Cell culture is one of major techniques in the life sciences. It is the general term used for the removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial environment conducive to their survival and/or proliferation. Basic environmental requirements for cells to grow optimally are: controlled temperature, substrate for cell attachment, and appropriate growth medium and incubator that maintains correct pH and osmolality. The most important and crucial step in cell culture is selecting appropriate growth medium for the *in vitro* cultivation. A growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms, cells, or small plants. Cell culture media generally comprise an appropriate source of energy and compounds which regulate the cell cycle. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. In addition to nutrients, the medium also helps maintain pH and osmolality.

Types of Cell Culture Media

Animal cells can be cultured either using a completely natural medium or an artificial/synthetic medium along with some natural products.

Table 1. Types of natural and artificial media.

	Media Type	Examples	Uses
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid	
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of bovine embryo and chick embryo	
	Clots	coagulants or plasma clots	
Artificial media	Balanced salt solutions	PBS, DPBS, HBSS, EBSS	Form the basis of complex media
	Basal media	MEM DMEM	Primary and diploid culture
	Complex media	RPMI-1640, IMDM	Supports wide range of mammalian cells

Natural Media

Natural media consist solely of naturally occurring biological fluids. Natural media are very useful and convenient for a wide range of animal cell culture. The major disadvantage of natural media is its poor reproducibility due to lack of knowledge of the exact composition of these natural media.

Artificial Media

Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors. Different artificial media have been devised to serve one or more of the following purposes: 1) immediate survival (a balanced salt solution, with specific pH and osmotic pressure); 2) prolonged survival

(a balanced salt solution supplemented with various formulation of organic compounds and/or serum); 3) indefinite growth; 4) specialized functions.

Artificial media are grouped into four categories:

Serum containing media

Fetal bovine serum is the most common supplement in animal cell culture media. It is used as a low-cost supplement to provide an optimal culture medium. Serum provides carriers or chelators for labile or water-insoluble nutrients, hormones and growth factors, protease inhibitors, and binds and neutralizes toxic moieties.

Serum-free media

Presence of serum in the media has many drawbacks and can lead to serious misinterpretations in immunological studies. A number of serum-free media have been developed. These media are generally specifically formulated to support the culture of a single cell type, such as Knockout Serum Replacement and Knockout DMEM from Thermo Fisher Scientific for stem cells, and incorporate defined quantities of purified growth factors, lipoproteins, and other proteins, which are otherwise usually provided by the serum. These media are also referred to as 'defined culture media' since the components in these media are known.

Chemically defined media

These media contain contamination-free ultra pure inorganic and organic ingredients, and may also contain pure protein additives, like growth factors. Their constituents are produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, specific amino acids, and fatty acids.

Protein-free media

Protein-free media do not contain any protein and only contain non-protein constituents. Compared to serum-supplemented media, use of protein-free media promotes superior cell growth and protein expression and facilitates downstream purification of any expressed product. Formulations like MEM, RPMI-1640 are protein-free and protein supplement is provided when required.

Basic Components of Culture Media

Culture media contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and available either as a powder or as a liquid form from commercial suppliers. The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Each component performs a specific function, as described below:

Buffering systems

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by an CO_2 incubator. Natural buffering system is low cost and non-toxic.

HEPES

Chemical buffering using a zwitterion, HEPES, has a superior buffering capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere. HEPES is relatively expensive and

toxic at a higher concentration for some cell types. HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light.

Phenol red

Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes color as pH is changed due to the metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture. However, there are certain disadvantages of using phenol red as described below:

1) Phenol red mimics the action of some steroid hormones, particularly estrogen. Thus it is advisable to use media without phenol red for studies using estrogen-sensitive cells like mammary tissue.

2) Presence of phenol red in some serum-free formulations interferes with the sodium-potassium homeostasis. This effect can be neutralized by the inclusion of serum or bovine pituitary hormone in the medium.

3) Phenol red interferes with detection in flow cytometric studies.

Inorganic salt

Inorganic salt in the media help to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions.

Amino Acids

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells can not

synthesize these by themselves. They ae required for the proliferation of cells and their concentration determines the maximum achievable cell density. L-glutamine, an essential amino acid, is particularly important. L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid, that, with time, converts to a form that cannot be used by cells, and should thus be added to media just before use. Caution should be used when adding more L-glutamine than is called for in the original medium formulation since its degradation results in the build-up of ammonia, and ammonia can have deleterious effect on some cell lines. L-glutamine concentrations for mammalian cell culture media can vary from 0.68 mM in Medium 199 to 4mM in Dulbecco's Modified Eagles's Medium. Invertebrate cell culture media can contain as much as 12.3 mM L-glutamine. Supplements like glutamax are more stable and can replace glutamine for long term culturing of slow cells.

Nonessential amino acids may also be added to the medium to replace those that have been depleted during growth. Supplementation of media with non-essential amino acids stimulates growth and prolongs the viability of the cells.

Carbohydrates

Carbohydrates in the form of sugars are the major source of energy. Most of the media contain glucose and galactose, however, some contain maltose and fructose.

Proteins and Peptides

The most commonly used proteins and peptides are albumin, transferrin, and fibronectin. They are particularly important in serum-free media. Serum is a rich source of proteins and includes albumin, transferrin, aprotinin, fetuin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells. The binding capacity of albumin makes it a suitable remover of toxic substances from the cell culture media.

Aprotinin is a protective agent in cell culture systems, stable at neutral and acidic pH and resistant to high temperatures and degradation by proteolytic enzymes. It has the ability to inhibit several serine proteases such as trypsin. Fetuin is a glycoprotein found in fetal and newborn serum at larger concentrations than in adult serum. It is also an inhibitor of serine proteases. Fibronectin is a key player in cell attachment. Transferrin is an iron transport protein that acts to supply iron to the cell membrane.

Fatty Acids and Lipids

They are particularly important in serum-free media as they are generally present in serum.

Vitamins

Many vitamins are essential for growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. Again serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.

Trace Elements

Trace elements are often supplemented to serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth [30]. These micronutrients are essential for many biological processes, e.g. the maintenance of the functionality of enzymes.

Media Supplements

The complete growth media recommended for certain cell lines requires additional components which are not present in the basal media and serum. These components, supplements, help

sustain proliferation and maintain normal cell metabolism. Although supplements like hormones, growth factors and signaling substances are required for normal growth of some cell lines, it is always best to take the following precautions: since the addition of supplement can change the osmolality of the complete growth media which can negatively affect the growth of cells, it is always best to recheck the osmolality after supplements are added. For most of the cell lines, optimal osmolality should be between 260 mOSM/kg and 320 mOSM/kg.

Shelf life of the growth media changes after the addition of supplements. Complete media containing protein supplement tend to degrade faster than basal media alone.

Antibiotics

Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria. Moreover, antibiotics can also interfere with the metabolism of sensitive cells.

Serum in Media

Serum is a complex mix of albumins, growth factors and growth inhibitors. Serum is one of the most important components of cell culture media and serves as a source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture. Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. Normal growth media often contain 2-10% of serum. Supplementation of media with serum serves the following functions:

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.

- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.
- It provides several binding proteins like albumin, transferrin, which can carry other molecules into the cell. For example: albumin carries lipids, vitamins, hormones, etc into cells.
- It also supplies proteins, like fibronectin, which promote attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they begin to divide.
- It provides protease inhibitors which protect cells from preolysis.
- It also provides minerals, like Na+, K+, Zn2+, Fe2+, etc.
- It increases the viscosity of medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffer.

Due to the presence of both growth factors and inhibitors, the role of serum in cell culture is very complex. Unfortunately, in addition to serving various functions, the use of serum in tissue culture applications has several drawbacks. Table 2 shows the advantages and disadvantages of using serum in the media.

Advantages of serum in media	**Disadvantages of serum in media**
Serum contains various growth factors and hormones which stimulates cell growth and functions.	Lack of uniformity in the composition of serum
Helps in attachment of the cells	Testing needs to be done to maintain the quality of each batch before using
Acts as a spreading factor	May contain some of the growth inhibiting factors
Acts as a buffering agent which helps in	Increase the risk of contamination

maintaining the pH of the culture media	
Functions as a binding protein	Presence of serum in media may interfere with the purification and isolation of cell culture products
Minimizes mechanical damages or damages caused by viscosity	

Preparation of Media

Culture medium is available in three forms from commercial suppliers:

1. Powdered form: it needs to be prepared and sterilized by the investigator.
2. Concentrated form: to be diluted by the investigator.
3. Working solution: to be used directly without further manipulation.

Powdered medium is the least expensive but needs to be sterilized. It is advisable to filter sterilize it prior to the addition of serum as the foaming that occurs in the presence of serum denatures the protein. Fetal bovine or horse sera can be added after filtration. Media should always be tested for sterility by placing it in a 37^oC CO_2 incubator for 72 hours prior to utilization to ensure that the lot is contamination-free. Medium should be stored at 4^oC. Since several components of medium are light-sensitive, it should be stored in dark.

Criteria for Selecting Media

Cell Lines

The choice of cell culture media is extremely important, and significantly affects the success of cell culture experiments. The selection of the media depends on the type of cells to be cultured and also the purpose of the culture and resources available in the laboratory. Different cell types have highly specific growth requirements, therefore, the most suitable media for each cell type must be determined experimentally. In general, it's always good to start with MEM for adherent

cells and RPMI-1640 for suspension cells. Table 3 describes commonly studied cell lines and recommended growth media.

Table 3. Commonly cell lines and recommended growth media

Cell Line	Morphology	Species	Medium	Applications
HeLa B	Epithelial	Human	MEM+ 2mM Glutamine+ 10% FBS + 1% Non Essential Amino Acids (NEAA)	Tumourigenicity and virus studies
HL60	Lymphoblast	Human	RPMI 1640 + 2mM Glutamine + 10-20% FBS	Differentiation studies
3T3 clone A31	Fibroblast	Mouse	DMEM + 2mM Glutamine +5% New Born Calf Serum (NBCS) + 5% FBS	Tumourigenicity and virus studies
COS-7	Fibroblast	Monkey	DMEM+ 2mM Glutamine + 10% FBS	Gene expression and virus replication studies
CHO	Epithelial	Hamster	Ham′s F12 + 2mM Glutamine + 10% FBS	Nutritional and gene expression studies
HEK 293	Epithelial	Human	EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 10% FBS	Transformation studies
HUVEC	Endothelial	Human	F-12 K + 10% FBS + 100 µg/ml Heparin	Angiogenesis studies
Jurkat	Lymphoblast	Human	RPMI-1640 + 10% FBS	Signaling studies

Primary Cell Culture

Primary cell culture provides unique and valuable research data, but most of the time cell number is the limitation. For such critical samples, especially from diseased human biopsies, a quality medium is required. Most of the life sciences companies are providing complete and ready to use, fully supplemented conditioned medium. This reduces the risk of contamination as well as save time, labor and money by eliminating the preparation steps and supplementation required. Moreover, all of these media are subjected to comprehensive quality control tests and each lot is routinely tested for growth promotion, absence of cytotoxicity, and physical parameters such as osmolality and pH level. Table 4 describes the recommended media provided by different companies for commonly used primary cells.

Cells	**Media**
Endothelial cells	EndoGRO-LS Complete Media Kit (EMD Millipore), HUVEC Basal Medium CB HUVEC (AllCells), Human Endothelial-SFM (Life Technologies), Endothelial Cell Medium (ScienCell Research Laboratories)
Bone marrow cells	MarrowMAX Bone Marrow Medium (Life Technologies), Bone Marrow Medium Plus (Sigma)
Glial cells	GIBCO® Astrocyte Medium
Epithelial cells	Epithelial cell medium (ScienCell Research Laboratory), EpiGRO primary epithelial cells (EMD Millipore)
T cells	Human StemXVivo Serum-Free T cell Base Media (R&D systems), Stemline T cell Expansion Medium (Sigma Aldrich)
Hematopoietic stem cells	StemPro-34 SFM (Life Technologies), MethoCult (STEMCELL Technologies, Inc)

Common Cell Culture Media

Most commonly used culture media include the following and are discussed in detail at Sigma, ATCC, and Life Technologies.

Eagle's Minimum Essential Medium (EMEM)

EMEM was among the first widely used media and was formulated by Harry Eagle from a simpler basal medium (BME). EMEM contains balanced salt solution, nonessential amino acids, and sodium pyruvate. It is formulated with a reduced sodium bicarbonate concentration (1500 mg/l) for use with 5% CO_2. Since EMEM is a non-complex medium, it is generally fortified with additional supplements or higher levels of serum making it suitable for a wide range of mammalian cells.

Dulbecco's Modified Eagle's Medium (DMEM)

DMEM has almost twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids. The original formulation contained 1,000 mg/L of glucose and was first reported for culturing embryonic mouse cells. A further variation with 4500 mg/L of glucose has been proved to be optimal for culture of various types of cells. DMEM is a basal medium and contains no proteins or growth promoting agents. Therefore, it requires supplementation to be a "complete" medium. It is most commonly supplemented with 5-10% Fetal Bovine Serum (FBS). DMEM utilizes a sodium bicarbonate buffer system (3.7 g/L) and therefore requires artificial levels of CO_2 to maintain the required pH. Powdered media is formulated without sodium bicarbonate because it tends to gas off in the powdered state. Powdered media requires the addition of 3.7 g/L of sodium bicarbonate upon dissolving it in water. DMEM was used initially for the culture of mouse embryonic stem cells. It has been found to be widely applicable in primary mouse and chicken cells, viral plaque formation and contact inhibition studies.

RPMI-1640

RPMI-1640 is a general purpose media with a broad range of applications for mammalian cells, especially hematopoietic cells. RPMI-1640 was developed at Roswell Park Memorial Institute (RPMI) in Buffalo, New York. RPMI-1640 is a modification of McCoy's 5A and was developed

for the long-term culture of peripheral blood lymphocytes. RPMI-1640 uses a bicarbonate buffering system and differs from the most mammalian cell culture media in its typical pH 8 formulation. RPMI-1640 supports the growth of a wide variety of cells in suspension and grown as monolayers. If properly supplemented with serum or an adequate serum replacement, RPMI-1640 has a wide range of applications for mammalian cells, including the culture of fresh human lymphocytes, fusion protocols, and growth of hybrid cells.

Ham's Nutrient Mixtures

These were originally developed to support the clonal outgrowth of Chinese hamster ovary (CHO) cells. There has been numerous modifications to the original formulation including Hams's F-12 medium, a more complex formulation than the original F-10 suitable for serum-free propagation. Mixtures were formulated for use with or without serum supplementation, depending on the type of cells being cultured.

Ham's F-10: It has been shown to support the growth of human diploid cells and white blood cells for chromosomal analysis.

Ham's F-12: It has been shown to support the growth of primary rat hepatocytes and rat prostate epithelial cells. Ham's F-12 supplemented with 25 mM HEPES provides more optimum buffering.

Coon's modification of Ham's F-12: It consists of almost two times the amount of amino acids and pyruvate as compared to F-12 and also includes ascorbic acid. It was developed for culturing hybrid cells produced by viral fusion.

DMEM/F12:

It is a mixture of DMEM and Ham's F-12 and is an extremely rich and complex medium. It supports the growth of a broad range of cell types in both serum and serum-free formulations.

HEPES buffer is included in the formulation at a final concentration of 15 mM to compensate for the loss of buffering capacity incurred by eliminating serum.

Iscove's Modified Dulbecco's Medium (IMDM)

IMDM is a highly enriched synthetic media well suited for rapidly proliferating, high-density cell cultures. IMDM is a modification of DMEM containing selenium, and has additional amino acids, vitamins and inorganic salts as compared to DMEM. It has potassium nitrate instead of ferric nitrate and also contains HEPES and sodium pyruvate. It was formulated for the growth of lymphocytes and hybridomas. Studies have demonstrated that IMDM can support murine B lymphocytes, hemopoietic tissue from bone marrow, B cells stimulated with lipopolysaccharide, T lymphocytes, and a variety of hybrid cells.

Table 5 describes different cells/cell lines which can be cultured using above mentioned media:

Media	**Tissue or cell line**
MEM	Chick embryofibroblast, CHO cells, embryonic nerve cells, alveolar type cells, endothelium, epidermis, fibroblast, glia, glioma, human tumors, melanoma
DMEM	Endothelium, fetal alveolar epithelial type II cells, cervix epithelium, gastrointestinal cells, mouse neuroblastoma, porcine cells from thyroid glands, ovarian carcinoma cell lines, skeleton muscle cells, sertoli cells, Syrian hamster fibroblast
RPMI-1640	T cells and thymocytes, hematopoietic stem cells, human tumors, human myeloid leukemia cell lines, human lymphoblastoid leukemia cell lines, mouse myeloma, mouse leukemia, mouse erythroleukemia, mouse hybridoma, rat liver cells
Nutrient mixture F-10 and F-12	Chick embryo pigmented retina, bone, cartilage, adipose tissue, embryonic lung cells, skeletal muscle cells

IMDM	Bone marrow, hematopoietic progenitor cells, human lymphoblastoid leukemia cell lines

Table 5. Common media and their applications

Optimization of Cell Culture Media

The complexity of composition of cell culture media provides many challenges to optimize individual components of media. Most of the classical culture media were devised for small-scale low-density cultures and often require serum as a key nutrient. However, in biotechnology industry where there is a need to sustain high cell densities and increase cellular productivity, development and optimization of culture media is very critical. Typically, media for the biotechnology industry are serum free and have much higher concentration of nutrients than classical media. Optimization of media requires the following parameters to be considered:

Product to be made

The type of product needed will determine the medium optimization strategy.

For the rapid generation of cell numbers, cell growth rate and viability are critical. So, cell culture media should support maximal cell growth and sustain cell viability at increasing cell densities.

For the production of virus, not just high cell densities are required but there must be abundant nutrients in the media to sustain virus replication after infection.

For the production of recombinant protein, high cell density is required. However, nutrients required for the cell growth can compete with those required for production of proteins. It is, therefore, very important to carefully determine the maximum cell densities a given medium can sustain for a required level of productivity. In addition, it is very important to consider that changes to the medium during optimization must not affect product quality.

Cell lines to be used

Different cell lines have different nutritional requirements because of difference in metabolism which dictates media optimization methods. The most common cell lines used in the biotechnology industries are CHO cells, BHK-21, hybridoma cells, myeloma cells, and normal diploid fibroblasts. Certain cell lines have specific nutritional requirements, such as cholesterol for NS0 myeloma cells. Normal diploid fibroblasts require attachment factors to adhere and spread out on a surface for growth. They grow to much lower densities and therefore do not need nutrients in high quantities. Hybridoma cells lines are generally highly dependent on glutamine. They typically lack a stationary phase after reaching a peak cell density and then decline rapidly in viability. Optimization of medium, thus, would reduce decline in viability and improve monoclonal antibody production.

Manufacturing process involved

Manufacturing process mode would not only affect the choice of cell culture medium but also approaches to optimization. Different manufacturing processes used are:

Batch Process: A single medium is used to sustain cell growth and productivity. Medium should therefore be rich in nutrients but remain in physiological limits of the cells.

Fed-batch: Several kinds of media are used over the course of the cell culture, depending on the stage of the process. A growth medium is designed in such a way that it has lower nutrient concentrations when cell densities are low during inoculation but maintain high rates of cell growth during culture scale-up and early production. A separate production medium which has increased nutrient concentrations over growth medium can also be used when the culture reaches production stage.

Challenges in Media Development

Cell culture media technology has advanced tremendously during the past few decades. Finding a good cell culture medium is very important for the overall performance of cell culture. Today's challenge is to develop sophisticated media that can be individually optimized for a range of cell cultures. Diversity of cell lines and involvement of large number of media components makes it very difficult. The fact that many of those components are interdependent on others because of the complexity of cellular metabolic pathways adds another layer of complexity.

Labome Survey of Cell Culture Media in Literature

Labome conducts systematic survey about reagents and instruments cited in formal publications. Labome curated over 750 articles with citations of cell culture media, as of August 2, 2015. Table 6 lists the major types of the media and the main suppliers.

DMEM medium

Invitrogen is one of major suppliers of DMEM medium. Invitrogen DMEM media were used to culture COS-1 monkey kidney fibroblast cell line in order to study the effect of the interaction between UBE1L and the PML domain for ISG15ylation on PML/RARalpha, to perform heterotopic grafting in order to show that specific myeloid cells derived from the yolk sac, to investigate the effect of a C-terminally tethered G protein alpha-subunit on the recycling rate and post-endocytic fate of the beta2AR receptor, to culture cells in order to investigate the role of transcription factor Ets-1 in the regulation of Natriuretic Peptide Receptor-A expression, to perform cell culture in order to show that natural variation in plants could be induced by chromatin silencing modulation, to perform cell culture in order to study the mechanism of the AMPK's regulation towards the circadian clock.

MEM medium

MEM (Minimum Essential Medium) can be used with a variety of suspension and adherent mammalian cells. Invitrogen was also the major supplier of MEM medium. Applications of their products range from ES cell culture to show proper organogenesis needs B-type lamins in mice

and T cells culture to confirm Bcl-3 is part of the pathway whereby adjuvants affect T cell lifespans, to study of the role of progestin-stimulated rapid PR signaling in the transcriptional regulation of target genes involved in breast cancer cell proliferation.

RPMI-1640 medium

Roswell Park Memorial Institute (RPMI)-1640 medium was originally developed to culture human leukemic cells. Invitrogen RPMI-1640 has been used for a variety of mammalian cells. It was used to culture cells in order to study the role of Bacteroides fragilis in establishing host-microbial symbiosis, to study the effect of the interaction between UBE1L and the PML domain for ISG15ylation on PML/RARalpha, to culture LNCaP cells in order to prove the CBP in drosophila experimental model system can corepress the transactivation of androgen receptor in pericentric heterochromatin.

DMEM/F12 medium

DMEM/F12 is a 1:1 mixture of DMEM and Ham's F-12. It is an extremely rich complex medium. Most of the DMEM/F12 media cited in this survey were provided by Invitrogen. They were used to support the growth of a wide range of cell types and study their biological characteristics, such as the regulation of PFK1 glycosylation on cancer cell growth, the role of Oct-4 expression in tumor malignancy, the role of Vsx2 in mediating mitogen signaling, the role of luteinizing hormone signaling in the early activation of the EGF network, the effect of topical immunosuppressive agents on the survival of cultivated allo-conjunctival equivalents, the role for CFTR in regulating NFkappaB mediated innate immune response and the culture of neuronal progenitor (NP) cells isolated from a human fetal brain.

Ham's F-12

Ham's F-12 Nutrient Mixture (F-12) has been used for serum-free growth of CHO cultures as well as serum-supplemented growth of other mammalian cells. Invitrogen offered a variety of F-12 modifications for a range of cell culture applications.

Neuralbasal medium

Neuralbasal medium is a basal medium that meets the special cell culture requirements of post-natal and adult neuronal cells. All the neuralbasal medium cited in the survey were provided by Invitrogen. It was used to grow neuronal cells from hippocampus, cortex and other regions of the brain.

Schneider's Drosophila medium

Schneider's Drosophila medium was originally designed for the growth of S2 cells from the fruit fly, *Drosophila melanogaster*.

McCoy's 5A medium

McCoy's 5A medium is a general purpose medium that supports the propagation of many types of cells. Invitrogen provided most of the McCoy's 5A medium in the surveyed cohort.

Addition of serum to the culture media has been an age-old practice. However, in recent years, certain serum-free media have been developed. It is worthwhile to know the disadvantages associated with the use of serum, and the advantages and disadvantages of serum-free media.

Disadvantages of serum in media:

Variable composition:

There is no uniformity in the composition of the serum. It is highly variable (source, batch, season, collection method, processing). Such differences in the composition significantly influence the cells in culture.

Quality control:

To maintain a uniform quality of the serum, special tests have to be performed with each batch of serum, before its use.

Contamination:

It is rather difficult to get serum totally free from all pathogens, particularly viruses.

Presence of growth inhibitors:

In general, the concentration of growth promoters in the serum is much higher than the inhibitors. But sometimes, the growth inhibitors such as TGF-P may dominate and inhibit cell proliferation.

Availability and cost:

There is a dependence on the cattle for the supply of serum. Hence the availability may be restricted on several occasions for political and economic reasons. Further, cost also is another factor for discouraging the use of serum.

Downstream processing:

The presence of serum in the culture medium interferes with the isolation and purification of cell culture products. For this reason, several additional steps may be required for the isolation of the desired product.

Advantages and Disadvantages of Serum-Free Media:

Advantages:

The limitations associated with the use of serum in the media (described above) are eliminated in the serum-free media. In addition, there are two more distinct advantages.

Selection of media with defined composition:

The main advantage of serum-free medium is to control growth of the cells as desired, with a well-defined medium. This is in contrast to the use of serum wherein the growth frequently proceeds in an uncontrolled fashion.

Regulation of differentiation:

It is possible to use a factor or a set of factors to achieve differentiation of cells with the desired and specialised functions.

Disadvantages:

Slow cell proliferation:

Most of the serum-free media are not as efficient as serum added media in the growth promotion of cells.

Need for multiple media:

A large number of serum-free media need to be developed for different cell lines. This may create some practical difficulties in a laboratory simultaneously handling several cell lines. Another limitation of serum-free medium is that a given medium may not be able to support the different stages of development even for a given cell line. Hence, sometimes separate media may be required even for the same cell line.

Purity of reagents:

The native serum does possess some amount of protective and detoxifying machinery that can offer a cleansing effect on the apparatus and reagents. And therefore, in the absence of serum, pure grade reagents and completely sterile apparatus should be used.

Availability and cost:

In general, the serum-free media are costlier than the serum added media. This is mainly due to the fact that many of the pure chemicals added to the serum-free media are themselves expensive. Further, the availability of serum-free media is also another limitation.

Development of Serum-Free Media:

While designing serum-free media, it is desirable to identify the various serum constituents and their quantities. The most important constituents of natural serum with reference to their use in cell cultures may be categorized as follows.

i. Growth regulatory factors e.g. PDGF, TGF-β.

ii. Cell adhesion factors e.g. vitamins.

iii. Essential nutrients e.g. vitamins, metabolites, minerals, fatty acids.

iv. Hormones e.g. insulin, hydrocortisone.

For replacing the serum and development of serum-free media, several constituents should supplement the media. Some highlights are given below.

Growth factors:

A large number of growth factors (nearly 100) that promote in vitro cell proliferation and differentiation have been identified. Besides the factors described already (above), some others are listed below.

i. Erythropoietin (EPO).

ii. Eye-derived growth factors (EDGF 1 and EDGF 2).

iii. Interleukins (IL-1, IL-2).

iv. Hepatocyte growth factor (HGF).

v. Brain-derived neurotrophic factor (BDNF).

vi. Phytohemagglutinin (PHA).

vii. Lipopolysaccharide (LPS).

The growth factors may act synergistically or additively with each other or with other factors (e.g. hormones, prostaglandins).

Almost all the growth factors are now commercially available for the preparation of serum-free media.

Hormones:

Growth hormone, insulin and hydrocortisone are the most commonly added hormones into the serum- free media. A combination of steroid hormones- hydrocortisone, estrogen, androgen and progesterone are used in formulating serum-free media for the maintenance of mammary epithelium.

Nutrients:

Addition of certain nutrients-choline, ethanol- amine, linoleic acid, iron, copper, selenium etc., are added in most of the serum-free media.

Proteins:

Bovine serum albumin (BSA) is the most commonly added protein. It promotes cell survival and growth.

Polyamines:

Putrescine is the most widely added polyamine to the serum-free media. Polyamines promote cellular growth and differentiation.

Protease inhibitors:

Addition of protease inhibitors (e.g. soy bean trypsin inhibitor) is done to the serum-free media for the trypsin-mediated subcultures.

Commonly Used Serum-Free Media:

Several types of serum-free media have been developed for different cell lines. A selected list of cell lines and the media is given in the Table 34.5.

Things to consider in serum-free culture

Overall, cells in serum-free culture are more sensitive to extremes of pH, temperature, osmolality, mechanical forces, and enzyme treatment.

Antibiotics

It is best not to use antibiotics in serum-free media. If you do, we recommend that you use 5- to 10-fold less than you would in a serum-supplemented medium. This is because serum proteins tend to bind a certain amount of the antibiotic added; without these serum proteins the level of antibiotic may be high enough to be toxic to certain cells.

Higher density

Cells must be in the mid-logarithmic phase of growth, with viability >90% prior to adaptation. Sequential adaptation may be necessary. Seeding cultures at a higher density than normal at each passage during SFM adaptation may help the process. Because some percentage of cells may not survive in the new culture environment, having more cells present will increase the number of

viable cells to further passage.

Clumping

Cell clumping often occurs during adaptation to SFM. We recommend that you gently triturate the clumps to break them up when passaging cells.

Morphology

It is not uncommon to see slight changes in cellular morphology during and after adaptation to SFM. As long as doubling times and viability remain good, slight changes in morphology should not be a reason for concern.

TABLE 34.5 A selected list of cell lines along with serum-free media

Cell line	*Medium*
Chick embryo fibroblasts	MCDB 202
Chinese hamster ovary (CHO)	MCDB 402
Human lung fibroblasts	MCDB 110
Human vascular endothelium	MCDB 131
Mammary epithelium	MCDB 170
Prostatic epithelium	WAJC 404
Bronchial epithelium	LHC 9
Fibroblasts	MCDB 202
3T3 cells	MCDB 402

MCDB

MCDB media were developed in the 1970s and 1980s, primarily by Ham and associates, for the low-protein and serum free growth of specific cell types using hormones, growth factors, trace

elements and/or low levels of dialyzed fetal bovine serum protein (FBSP). Each MCDB medium was formulated (quantitatively and qualitatively) to provide a defined and optimally balanced nutritional environment that selectively promoted the growth of a specific cell line.

- MCDB 105 and 110 are modifications of MCDB 104 medium, optimized for long-term survival and rapid clonal growth of human diploid fibroblast-like cells (WI-38, MRC-5, IMR-90) and low passaged human foreskin fibroblasts using FBSP, hormone, and growth factor supplements.

MCDB 151, 201 and 302 are modifications of Ham's nutrient mixture F-12, designed for the growth of human keratinocytes, clonal growth of chicken embryo fibroblasts (CEF) and Chinese hamster ovary (CHO) cells using low levels of FBSP, extensive trace elements or no serum protein.

LHC serum-free media are the most widely used products for culture of bronchial epithelial cells. For maximum flexibility and convenience, we offer these products with or without supplementation.

LHC Basal Medium (1X), liquid

Applications:

Formulated for the growth of bronchial epithelial cells with further supplements.

Recommended storage conditions:

2° to 8°C.

Shelf life:

12 months

LHC-8 Medium and LHC-9 Medium
Applications:

Engineered specifically for the growth of bronchial epithelial cells such as IB3-1, S9, and C38, BEAS-2B, BZR, BBM, Het-1A, NHBE (normal human bronchial epithelial cells), and BBE (bovine bronchial epithelial cells) for asthma, allergy, lung cancer, cystic fibrosis, pulmonary, and esophageal cancer research without further supplements.

Performance and quality testing:

Performance tested in a growth assay using BEAS-2B cells.

Recommended storage conditions:

-5°C to -20°C.

Specifications

Cell Type:	Epithelial Cells (Bronchial)
Classification:	Serum-Free
Form:	Liquid
Glutamine:	L-Glutamine
Phenol Red Indicator:	Phenol Red
Product Size:	500 Ml
Serum Level:	Serum-Free
Species:	Cattle/Bovine, Human
Green Features:	Sustainable packaging
Shipping Condition:	Dry Ice

Contents & storage

Store in freezer (-5 to -30°C) and protect from light.

Possible Questions

2 Marks

1. Define culture media.
2. What is balances salt solutions
3. What is the role of CO_2 in media

8 Marks

1. Explain the types of media,
2. What are the physical, chemical and metabolic functions of different constituents of culture medium
3. What is serum and serum free media. Explain it with advantages and disadvantage.
4. Explain protein free defined media and their applications

UNIT-II

SYLLABUS

Cell culture: Types, disaggregation of tissue, primary culture, established culture; suspension culture, organ culture, three dimensional culture and tissue engineering, feeder layers; cell synchronization; cryopreservation. Biology and characterization of cultured cells, tissue typing; cell – cell interaction; measuring parameters of growth; measurement of cell death – apoptosis and its determination.

Tissue Culture

Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semisolid medium that supplies the nutrients essential for survival and growth. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called **Organ Culture**. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, it is called **Cell Culture**.

Primary Culture

Primary Culture When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a **Primary Culture**. There are two basic methods for doing this. First, for **Explant Cultures**, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow. The **second**, more widely used method speeds up this process by adding digesting (proteolytic) enzymes, such as **trypsin** or

collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide. This method is called **Enzymatic Dissociation**

Techniques for Primary Culture:

Among the various techniques devised for the primary culture of isolated tissues, three techniques are most commonly used:

1. Mechanical disaggregation.
2. Enzymatic disaggregation.
3. Primary explant technique.

Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique

Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin. Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization

Warm trypsinization

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice). The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

Cold trypsinization

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique. After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispersed by repeated pi-petting's. The

dissociated cells can be counted, appropriately diluted and then used. The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

Disaggregation by collagenase:

Collagen is the most abundant structural protein in higher animals. It is mainly present in the extra - cellular matrix of connective tissue and muscle. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the disaggregation of several tissues (normal or malignant) that may be sensitive to trypsin. Highly purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase. The important stages in collagenase dis aggregation, depicted in are briefly described hereunder.

The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated. Collagenase disaggregation has been successfully used for human brain, lung and several other epithelial tissues, besides various human tumors, and other animal tissues. Addition of another enzyme hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation. Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. This can be done by per-fusing the whole organ in situ. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g. pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

Primary Explant Technique:

The primary explant technique was, in fact the original method, developed by Harrison in 1907. This technique has undergone several modifications, and is still in use. The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial out growth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel. The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation. However, are not suitable for small amounts of tissues, as there is a risk of losing the cells. The limitation of explant technique is the poor adhesiveness of certain tissues to the growth surface, and the selection of cells in the outgrowth. It is however, observed that the primary explants technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells, glial cells.

Separation of Viable and Non-Viable Cells:

It is a common practice to remove the non-viable cells while the primary culture is prepared from the disaggregated cells. This is usually done when the first change of the medium is carried out. The very few left over non-viable cells get diluted and gradually disappear as the proliferation of viable cells commences. Sometimes, the non-viable cells from the primary cultures may be removed by centrifugation. The cells are mixed with ficoll and sodium metrizoate, and centrifuged. The dead cells form a pellet at the bottom of the tube.

What Are Cultured Cells Like?

Once in culture, cells exhibit a wide range of behaviors, characteristics and shapes. Some of the more common ones are described below.

Cell Culture Systems

Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (**Monolayer Culture Systems**) or floating free in the culture medium (**Suspension Culture Systems**). Monolayer cultures are usually grown in tissue culture treated dishes, T-flasks, roller bottles, or multiple well plates, the choice being based on the number of cells needed, the nature of the culture environment, cost and personal preference. Suspension cultures are usually grown either:

1. In magnetically rotated spinner flasks or shaken Erlenmeyer flasks where the cells are kept actively suspended in the medium;

2. In stationary culture vessels such as T-flasks and bottles where, although the cells are not kept agitated, they are unable to attach firmly to the substrate. Many cell lines, especially those derived from normal tissues, are considered to be **Anchorage Dependent,** that is, they can only grow when attached to a suitable substrate. Some cell lines that are no longer considered normal (frequently designated as **Transformed Cells)** are frequently able to grow either attached to a substrate or floating free in suspension; they are **Anchorage-Independent.** In addition, some normal cells, such as those found in the blood, do not normally attach to substrates and always grow in suspension.

Established cell line

Cells that demonstrate the potential for indefinite subculture in vitro.

Three dimensional tissue cultures and tissue engineering – Basic principles

Tissue damages caused by mechanical injuries or diseases are frequent causes of morbidity and mortality. Tissue injuries are normally repaired by "built-in" regeneration mechanisms. However, if the tissue regeneration process malfunctions or the extent of the injury is too large,

organ transplant can potentially be the only solution. Lack of transplantable organs when other therapies have all been exhausted adversely affects the quality and length of patients' life, and is severe financial burden on the individual and society. Tissue injury associated diseases would become treatable using targeted tissue-regeneration or transplantation therapies. To provide tissues for therapy or for research to study tissue specific physiological mechanisms and diseases processes, the discipline of tissue engineering has evolved.

Originally, tissue engineering was categorized as a sub-field of engineering and bio-materials, but having grown in scale and significance tissue engineering has become a discipline of its own. To regenerate or even to re-create certain parts of the human body, tissue engineering uses combinations of various methods of cell culture, engineering, bio-materials and suitable biochemical and biophysical factors. While most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions of whole tissues including bone, cartilage, blood vessels, skin, etc.

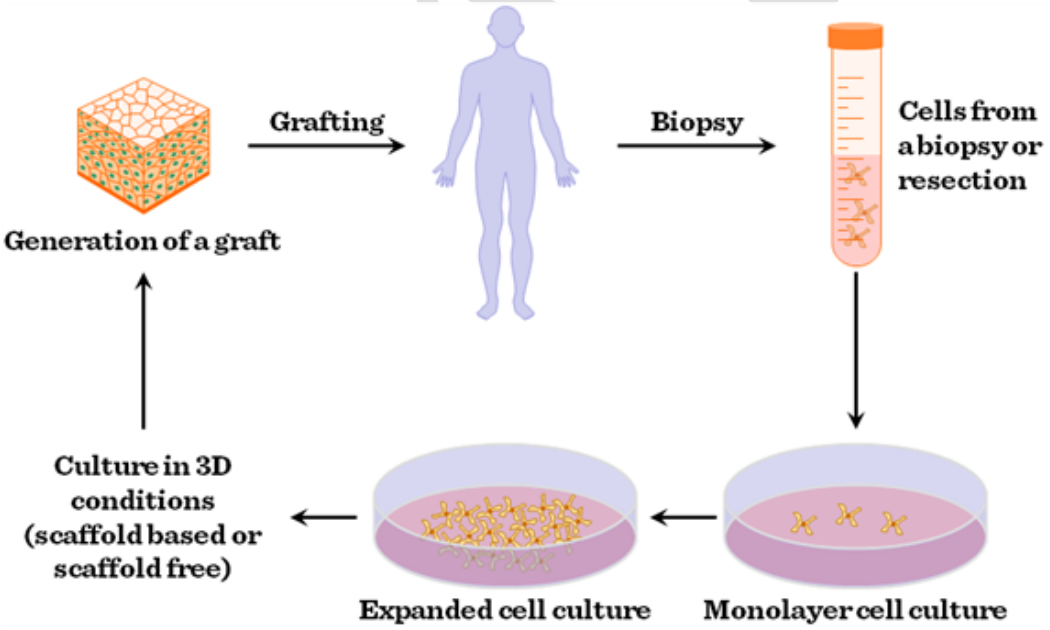


Figure 2.1: Basic principles of tissue engineering

Tissues created in vitro frequently originate from embryonic or adult cells. Furthermore, in vitro generated tissues often need certain mechanical support and complex manipulation to achieve the required structural and physiological properties for proper functioning. To achieve complex tissue structures, conventional cell cultures (Figure 2.2) where cells grow as monolayers in two-dimension (2D) can no longer be used.

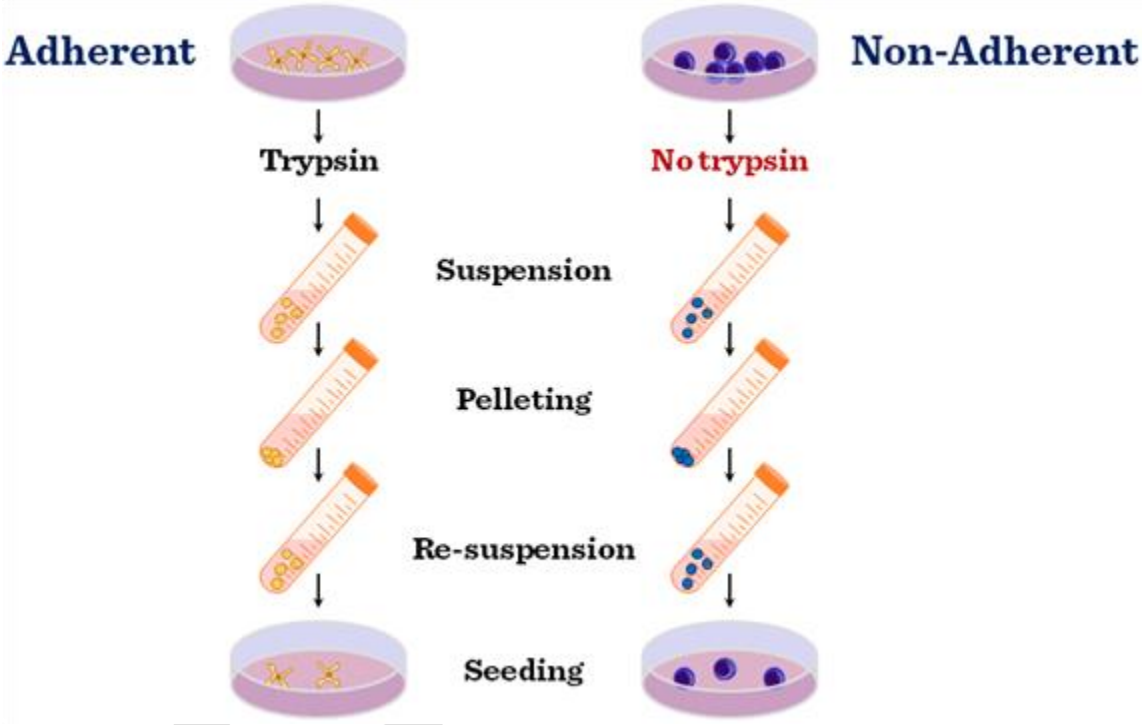


Figure 2.2: 2D tissue cultures

Particularly, as in monolayer cultures stretched cells form a single layer only network, that is incapable to perform complex functions. In tissue engineering, traditional cell culture technology is replaced by three-dimensional (3D) cell cultures (Figure 2.3) where cultured cells assume a more natural morphology and physiology.

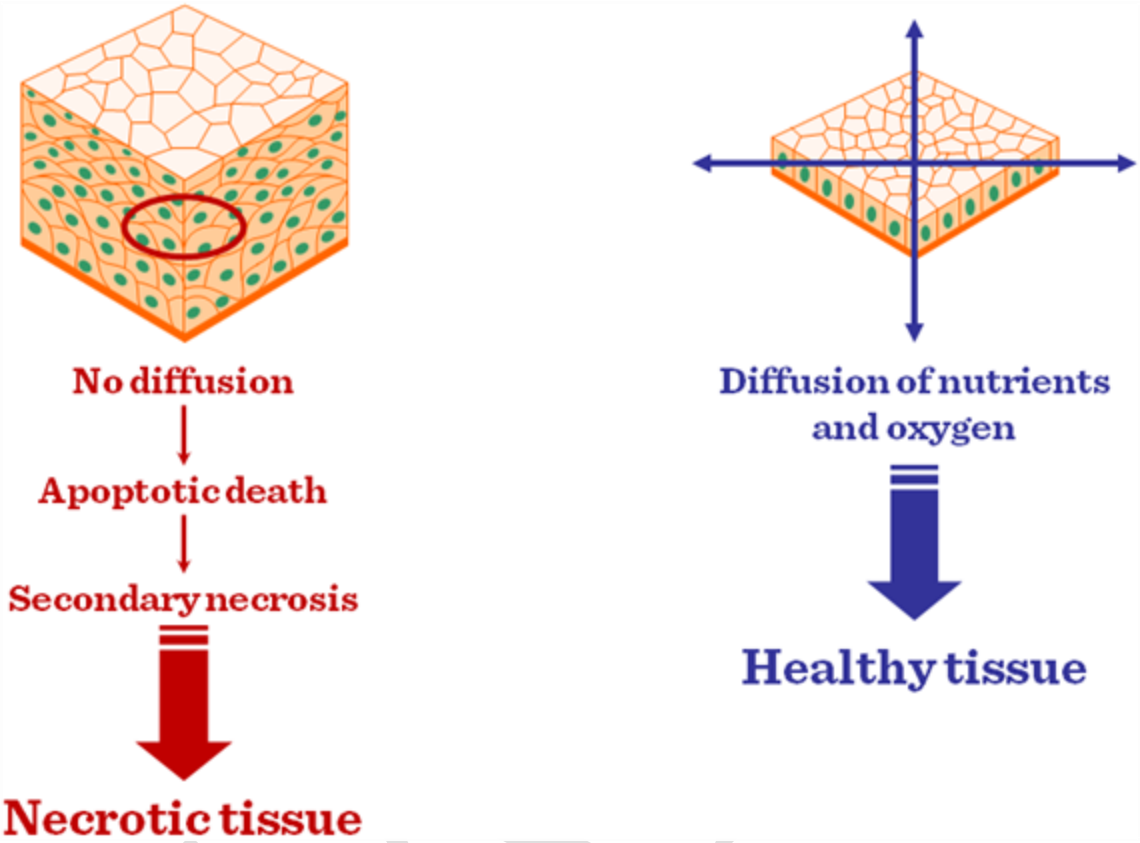


Figure 2.3: 3D tissue cultures

Various three dimensional tissue culture technologies have developed as tissue engineering gained impetus in medical research and therapy. Three dimensional culture technologies frequently apply various bio-materials where cells are provided with the necessary interactions to form the required tissue or organ.

If tissues are not needed immediately, both differentiated adult primary cells as well as adult and embryonic stem cells can be stored in liquid nitrogen, below -150°C.

3D cell culture: comparing 3D cell culture advantages and downsides with 2D cell culture

As you may already be aware of, there are different type of 3D cell culture, with each kind of them offering different advantages and drawbacks. Unlike 2D cell culture, 3D cell culture facilitate cell differentiation and tissue organization by using micro-assembled structures and a complex environmental parameters. In fact, in a 3D environment, **cells tend to be more subjected to morphological and physiological changes** contrary to those grown in a 2D environment. This can mostly be explain by the structuring role and the influence of the scaffold that guide the cells behavior. Researchers have found that **the geometry and composition of this cellular support can not only influence genes expression but also enhance cell-cell communication**. For instance, some genes promoting cell proliferation are repressed in a 3D cell culture, hence avoiding the anarchic proliferation encountered in 2D cell culture.

3D cell culture also grants the possibility to **grow simultaneously two different cellular populations with co-cultures accurately reproducing cellular functions observed within a tissue unlike co-cultures based on 2D cell culture**. Interactions existing between cells of interest and others cell are obviously key element in cell functions. That's the reason why studies focusing on stromal cell (organ connective cell tissues) that play an important part in cancer have been conducted. Finally, using 3D cell culture **make it easier to control and monitor the growing cells micro-environment parameters** (temperature, chemical gradients, oxygen rate, pH, etc.) to a certain extent while remaining as close to reality as possible thanks to micro-engineering (microfluidic).

One must bear in mind that 3D cell culture is a relatively new technique that **researchers have not yet fully grasped the underlying phenomenon and implications**. Unfortunately, this culture method presents some noticeable downsides that would most likely be overcome by technological advances. First, **some scaffold matrices incorporate compounds from animal or others unwanted sources** (virus, soluble factors) that could interfere with the cell culture. Some other matrices provide good cell adherence, making **cell removal all the more difficult**. Beside, while 3D cell culture could be a cost saving technique that would skip the animal drug testing

step in drugs trials, developing automation and reproducible applications still remains a **very costly and meticulous process**.

SCAFFOLDS TYPES IN 3D CELLS CULTURES

Scaffolds are key supporting elements in 3D cell culture , and depending on the conditions and intended goals, different kinds of scaffolds are currently available.

Scaffold-based 3D cell culture technique

As pointed out above, scaffolds can be convenient supports for 3D cell culture. Due to their porosity, scaffolds facilitate oxygen, nutriment and waste transportation. Thus, C**ells can proliferate and migrate within the scaffold web to eventually adhere on it**. As they keep growing, the maturing cells end up interacting with each other and will eventually turn into structures closed to the tissues they were initially originated from. Most of the time, those aggregates are presented as heterogeneous-sized spheres called spheroids: that's the cell structure generally employed for drug screening and any other 3D cell culture application. Finally, 3D cell culture that use scaffolds offer bigger surface and are generally larger than those not relying on this support.

2.1.1 Scaffolds: categories and general composition

Depending on the cell types handled, the adequate scaffolds possessing suitable properties and shapes must be associated with. **The scaffold layout should match the tissue of interest**, reproducing its structure, scale (macro, micro, Nano) and function. However, the bigger and the more complex a scaffold is, the harder extraction for analysis purpose becomes. Besides, to avoid any hindrances (immunity system, fibrosis, weak growth), no matter the category considered, **the scaffold handled must provide cell growth support and present biocompatibility properties**.

Two different scaffold categories can be found: on the one hand, there are ***in vitro*** **3D scaffolds** for cell culture and experimental applications (drug and cosmetic testing), on the other hand **biomedical engineering scaffolds** are selected as support for tissues regeneration applications. In the late category, the scaffold can either be definitively implanted to help tissue reconstruction, either be removed or biodegraded after fulfilling its purpose.

Scaffold developed can be hydrogels, membranes (or tube) and **3D matrices**. Materials such as metals, glasses and ceramics can constitute a scaffold though polymers, synthetic or natural derived, are preferably used for an easier control of their chemical and structural surface properties.

2.1.2. Hydrogels scaffolds

Gels, materials showing good mechanical properties, are **one of the most used scaffold** since they present a tissue-like stiffness and perfectly mimic the extracellular matrix (ECM) in a certain extent. In fact, like any other scaffold, this porous material **acts as a rich extracellular matrix that can store nutrients and soluble factors** such as cytokines and growth factors which can navigate through the gel. These soluble factors are secreted by cells that can henceforth communicate otherwise than direct contact.

This substitute to native *in vivo* ECM indeed contain an import amount of water and natural biomolecules such as alginate, gelatin, hyaluronic acid, agarose, laminin, collagen or fibrin. The gelling mechanism to solidify a gel precursor can sometime be tricky, making the preparation and manipulation of gels a difficult task.

As you may know, **synthetic and natural biopolymer can also be used as gel for 3D cell culture**. Depending on the experimental conditions and the intended goal, different kinds of polymer can be found, ranging from inert to biodegradable (polyester, polyethylene glycol,

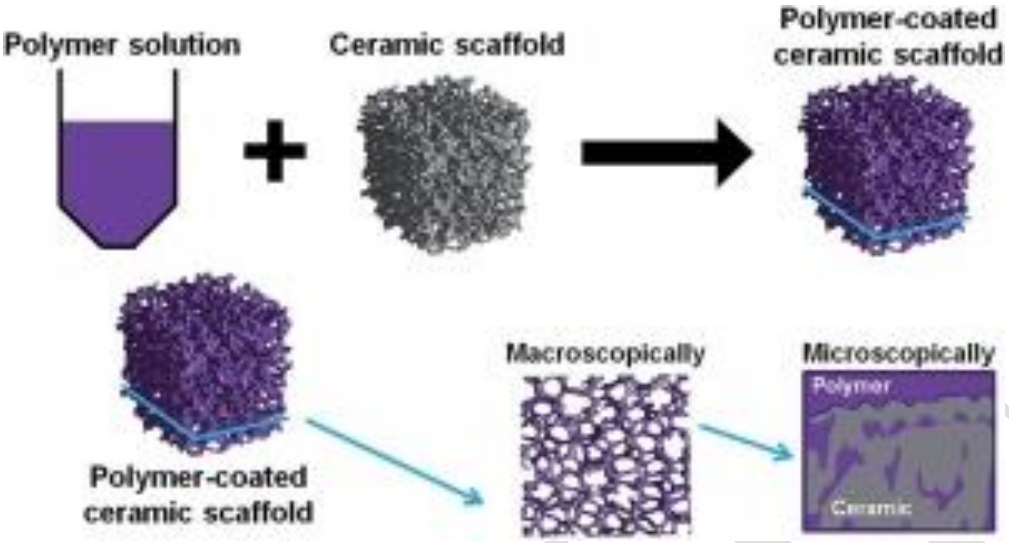
polyamide, polyglycolic acid, polylactic acid). Polymers are easier to manipulate, offering better and wider possibilities to accurately build a scaffold.

2.1.3. Other types of scaffold

Ceramics nanofibers scaffold

As previously mentioned, excluding hydrogels, there are a few other kinds of scaffold that can be found, though the vast majority of them are mostly used as tissue engineering scaffolds. One of the materials, bioglass or bioceramic, is a bioresorbable material that improve the regeneration activity of a nascent tissue. On the other hand, porous metallic scaffolds mostly made of titanium (Ti) and tantalum (Ta), have been designed since metals have high compressive strengths and above all excellent fatigue resistance.

Non gel Polymer scaffold commonly used are natural polymers for tissue engineering such as collagen, fibrin, alginate, silk, hyaluronic acid, and chitosan. As for synthetic polymers, there is poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and polycaprolactone (PCL). These polymers are preferentially employed since they produce monomers that are easily removed by the natural physiological pathway when implanted. Lastly, composites are also used to build scaffolds. They are made of two or more distinctly different materials (ceramics combined with polymers for instance) developed to takes advantages of both materials properties to meet mechanical and physiological requirements.



Fabrication process and microscopic view of a composite scaffold (from pubs.rsc.org)

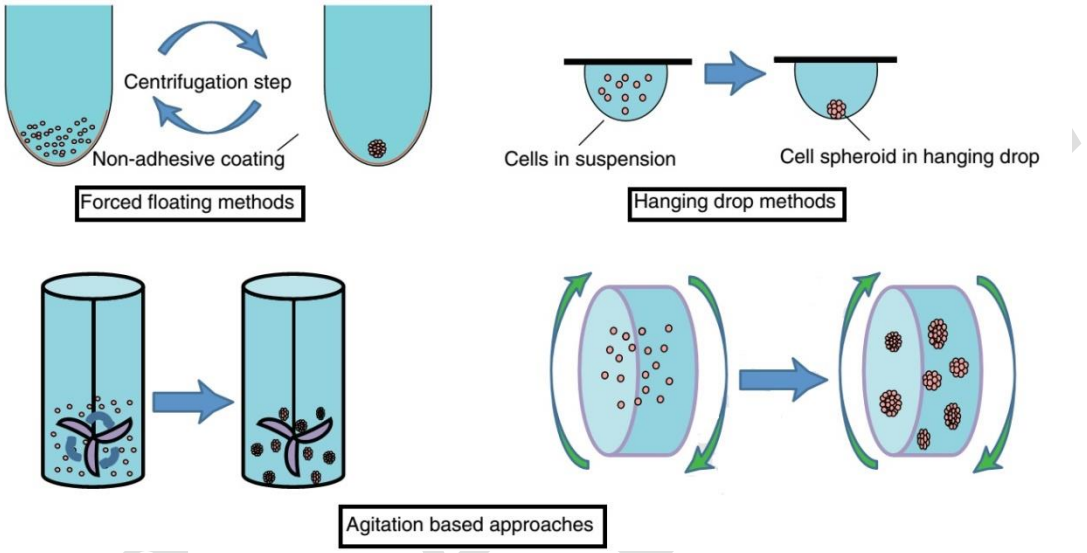
2.2. Scaffold-free 3D cell culture techniques

To generate spheroids, cells aggregates serving as good physiological models, 3D cultures that don't rely on solid supports (ECM molecules or biomaterials) can also be made. Spheroids obtained using this technique are most of time smaller and less resistant. The main scaffold-free 3D cells cultures techniques are the forced-floating method, the hanging drop method and the agitation based method.

Scaffold free techniques include **forced-floating methods** that use low adhesion polymer-coated well-plates. Spheroid are generated by filling those well-plates with a cell suspension after centrifugation.

Hanging drop methods, scaffold free techniques, consists in placing a cell suspension aliquot inside a MicroWell MiniTray (Nunc). By inverting the plates (trays), aliquots become droplets presenting cell aggregates on it tips and thus creating compact and homogeneous spheroids.

Last but not least, **agitation based approaches** using bioreactor can also be a simple alternative method to obtain three-dimensional spheroids. A cell suspension placed into a rotating bioreactor gradually turns isolated cells into aggregates that cannot adhere to the container wall due to the continuous stirring. As a result, a broad range of non-uniform spheroids are eventually generated.



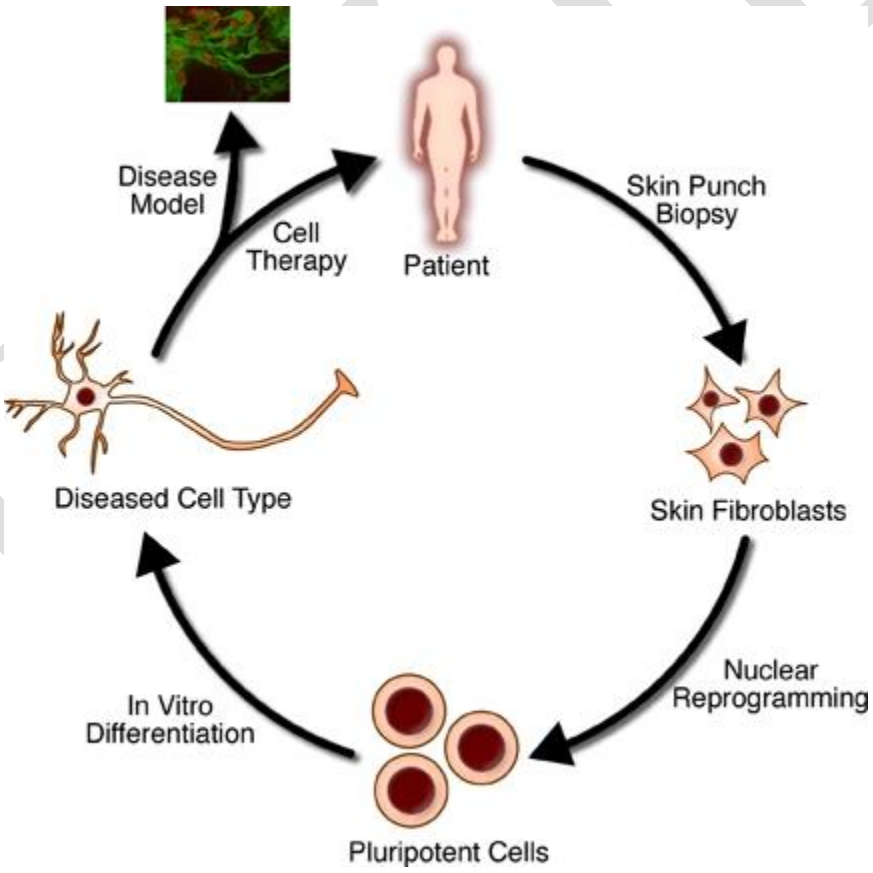
Scaffold-free 3D cell culture techniques

Tissue engineering, scientific field concerned with the development of biological substitutes capable of replacing diseased or damaged tissue in humans. The term *tissue engineering* was introduced in the late 1980s. By the early 1990s the concept of applying engineering to the repair of biological tissue resulted in the rapid growth of tissue engineering as an interdisciplinary field with the potential to revolutionize important areas of medicine.

Tissue engineering integrates biology with engineering principles and synthetic materials to develop substitute tissues capable of replacing diseased or damaged tissues in humans. Tissue

engineering has played an important role in improving the success of skin graft surgeries for complex wounds such as burns.

Tissue engineering integrates biological components, such as cells and growth factors, with engineering principles and synthetic materials. Substitute tissues can be produced by first seeding human cells onto scaffolds, which may be made from collagen or from a biodegradable polymer. The scaffolds are then incubated in mediums containing growth factors, which stimulate the cells to grow and divide. As cells spread across the scaffold, the substitute tissue is formed. This tissue can be implanted into the human body, with the implanted scaffold eventually being either absorbed or dissolved.



Examples of tissues that are candidates for tissue engineering include skin, cartilage, heart, and bone. The production of skin substitutes has played an important role in improving the success of skin graft surgeries, especially for complex wounds such as burns. Substitute tissues of the renal system, including urinary bladders and urethras, have also been engineered and transplanted successfully, thereby broadening therapeutic opportunities for complicated renal disorders. Scaffolds and bioartificial tissues are being investigated for their use in the development of functioning bioartificial limbs; the first such limb to be successfully developed—a rat leg with functioning muscles and veins—was reported in 2015.

Bioartificial limb: A bioartificial rat limb shown suspended in a bioreactor that contains a nutrient solution and electrical stimulation to support and promote the growth of new tissue. *Bernhard Jank, MD, Ott Laboratory/Massachusetts General Hospital Center for Regenerative Medicine*

Tissue engineering evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs. Artificial skin and cartilage are examples of engineered tissues that have been approved by the FDA; however, currently they have limited use in human patients.

Regenerative medicine is a broad field that includes tissue engineering but also incorporates research on self-healing – where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs. The terms "tissue engineering" and "regenerative medicine" have become largely interchangeable, as the field hopes to focus on cures instead of treatments for complex, often chronic, diseases.

This field continues to evolve. In addition to medical applications, non-therapeutic applications include using tissues as biosensors to detect biological or chemical threat agents, and tissue chips that can be used to test the toxicity of an experimental medication.

What is Tissue Engineering?

How do tissue engineering and regenerative medicine work?

Cells are the building blocks of tissue, and tissues are the basic unit of function in the body. Generally, groups of cells make and secrete their own support structures, called extra-cellular matrix. This matrix, or scaffold, does more than just support the cells; it also acts as a relay station for various signaling molecules. Thus, cells receive messages from many sources that become available from the local environment. Each signal can start a chain of responses that determine what happens to the cell. By understanding how individual cells respond to signals, interact with their environment, and organize into tissues and organisms, researchers have been able to manipulate these processes to mend damaged tissues or even create new ones.

The process often begins with building a scaffold from a wide set of possible sources, from proteins to plastics. Once scaffolds are created, cells with or without a "cocktail" of growth factors can be introduced. If the environment is right, a tissue develops. In some cases, the cells, scaffolds, and growth factors are all mixed together at once, allowing the tissue to "self-assemble."

Another method to create new tissue uses an existing scaffold. The cells of a donor organ are stripped and the remaining collagen scaffold is used to grow new tissue. This process has been used to bioengineer heart, liver, lung, and kidney tissue. This approach holds great promise for using scaffolding from human tissue discarded during surgery and combining it with a patient's own cells to make customized organs that would not be rejected by the immune system.

How do tissue engineering and regenerative medicine fit in with current medical practices?

A biomaterial made from pigs' intestines which can be used to heal wounds in humans. When moistened, the material, which is called SIS, is flexible and easy to handle.

Currently, tissue engineering plays a relatively small role in patient treatment. Supplemental bladders, small arteries, skin grafts, cartilage, and even a full trachea have been implanted in patients, but the procedures are still experimental and very costly. While more complex organ tissues like heart, lung, and liver tissue have been successfully recreated in the lab, they are a long way from being fully reproducible and ready to implant into a patient. These tissues, however, can be quite useful in research, especially in drug development. Using functioning human tissue to help screen medication candidates could speed up development and provide key tools for facilitating personalized medicine while saving money and reducing the number of animals used for research.

What are NIH-funded researchers developing in the areas of tissue engineering and regenerative medicine?

Research supported by NIBIB includes development of new scaffold materials and new tools to fabricate, image, monitor, and preserve engineered tissues. Some examples of research in this area are described below.

- ***Controlling stem cells through their environment:***
  For many years, scientists have searched for ways to control how stems cells develop into other cell types, in the hopes of creating new therapies. Researchers have grown pluripotent cells—stem cells that have the ability to turn into any kind of cell—in different types of defined spaces and found that this confinement triggered very specific gene networks that determined the ultimate fate for the cells. Most other medical research on pluripotent stem cells has focused on modifying the combination of growth solutions in which the cells are placed. The discovery that there is a biomechanical element to controlling how stem cells transform into other cell types is an important piece of the puzzle as scientists try to harness stems cells for medical uses.

- ***Implanting human livers in mice:***
  Researchers have engineered human liver tissue that can be implanted in a mouse. The mouse retains its own liver as well, and therefore its normal function-but the added piece of engineered human liver can metabolize drugs in the same way humans do. This allows researchers to test susceptibility to toxicity and to demonstrate species-specific responses that typically do not show up until clinical trials. Using engineered human tissue in this way could cut down on the time and cost of producing new drugs, as well as allow for critical examinations of drug-drug interactions within a human-like system.

- ***Engineering mature bone stem cells:***
  Researchers has been able to take stem cells all the way from their pluripotent state to mature bone grafts that could potentially be transplanted into a patient. Previously, investigators could only differentiate the cells to a primitive version of the tissue which was not fully functional. Additionally, the study found that when the bone was implanted in immunodeficient mice there were no abnormal growths afterwards—a problem that often occurs after implanting stem cells or bone scaffolds alone.

- ***Using lattices to help engineered tissue survive:***
  Currently, engineered tissues that are larger than 200 microns (about twice the width of a human hair) in any dimension cannot survive because they do not have vascular networks (veins or arteries). Tissues need a good "plumbing system"—a way to bring nutrients to the cells and carry away the waste—and without a blood supply or similar mechanism, the cells quickly die. Ideally, scientists would like to be able to create engineered tissue with this plumbing system already built in. Researcher is working on a very simple and easily reproducible system to solve this problem: a modified ink-jet printer that lays down a lattice made of a sugar solution. This solution hardens and the engineered tissue

(in a gel form) surrounds the lattice. Later, blood is added which easily dissolves the sugar lattice, leaving pre-formed channels to act as blood vessels.

- ***New hope for the bum knee:***
  Until now, cartilage has been very difficult, if not impossible, to repair due to the fact that cartilage lacks a blood supply to promote regeneration. There has been a 50% long-term success rate using microfracture surgery in young adults suffering from sports injuries, and little to no success in patients with widespread cartilage degeneration such as osteoarthritis. An tissue engineer has developed a biological gel that can be injected into a cartilage defect following microfracture surgery to create an environment that facilitates regeneration. However, in order for this gel to stay in place within the knee, researchers also developed a new biological adhesive that is able to bond to both the gel as well as the damaged cartilage in the knee, keeping the newly regrown cartilage in place. The gel/adhesive combo was successful in regenerating cartilage tissue following surgery and decreased pain at six months post-surgery. In contrast, the majority of microfracture patients, after an initial decrease in pain, returned to their original pain level within six months.

- ***Regenerating a new kidney:***
  The ability to regenerate a new kidney from a patient's own cells would provide major relief for the hundreds of thousands of patients suffering from kidney disease. Experimenting on rat, pig and human kidney cells, researchers broke new ground on this front by first stripping cells from a donor organ and using the remaining collagen scaffold to help guide the growth of new tissue. To regenerate viable kidney tissue, researchers seeded the kidney scaffolds with epithelial and endothelial cells. The resulting organ tissue was able to clear metabolites, reabsorb nutrients, and produce urine both *in vitro* and *in vivo* in rats. This process was previously used to bioengineer heart, liver, and lung tissue. The creation of transplantable tissue to permanently replace kidney function is a

leap forward in overcoming the problems of donor organ shortages and the morbidity associated with immunosuppression in organ transplants.

Feeder layer

A population of connective tissue cells that are used to nourish cultured tissue cells in the laboratory. The feeder cell layer is often derived from mouse fibroblasts. Feeder cells supply metabolites to the cells they support, do not grow or divide, and can be inactivated by gamma irradiation.

Cultures of growth-arrested feeder cells have been used for years to promote cell proliferation, particularly with low-density inocula. Basically, feeder cells consist in a layer of cells unable to divide, that provides extracellular secretions to help another cell to proliferate. It differs from a co-culture system because only one cell type is capable to proliferate. It is known that feeder cells support the growth of target cells by releasing growth factors to the culture media, but this is not the only way that feeder cells promote the growth of target cells. In this work we discuss what are the different mechanisms of action of feeder cells, tackling questions such as why for some cell cultures the presence of feeder cell layers is mandatory, while in some other cases the growth of target cells can be achieved just with conditioned medium. Different treatments to avoid feeder cells to proliferate are revised, not only the classical treatments as mitomycin or gamma-irradiation, but also not so common treatments as electric pulses or chemical fixation. Regenerative medicine has been gaining importance in recent years as a discipline that moves biomedical technology from the laboratory to the patients. In this context, human stem and pluripotent cells play an important role, but feeder cells presence is necessary for these progenitor cells to grow and differentiate.

Feeder Layer Tissue Culture

Swiss 3T3 cells (a TRAP positive feeder cell line that grows well in serum containing media such as DMEM/F12 plus calf or fetal calf serum)

Preparing the Swiss 3T3 cells for use as a feeder layer :

1.) Once plate is confluent, treat with 10 µg/mL mitomycin C (stock 0.5 mg/mL, dissolved in H_2O) in complete medium for 2 hrs at 37^oC.

2.) After treatment, wash plate with pre-warmed Solution A (or serum-free medium) at least 3 times, aspirating after each wash.

3.) Split the 3T3 cells by adding trypsin/EDTA; aspirate after 30-40 sec, incubate another 30 sec at RT, tap the plate to dislodge the cells, and resuspend them in complete medium.

4.) Spin the cell suspension down to obtain a cell pellet.

5.) Pellets can be used as feeder layers immediately, or can be saved for later use.

a) For immediate use :
- Wash pellet in pre-warmed Sol'n A and spin down
- Resuspend in DMEM/F12
- Plate the 3T3 cells 1:3 and mix with an appropriate number of epithelial cells (low density, 250k for a 100mm plate) in DMEM/F12

b) For next day use :
- Wash pellet in pre-warmed Sol'n A and spin down
- Resuspend in complete medium
- Plate 1:3 in complete medium and allow to attach overnight
- Wash plate with pre-warmed Sol'n A x2 and put on DMEM/F12
- Plate epithelial cells (low density, 250k for a 100mm plate)

c) For later use :
- Wash pellet in pre-warmed Sol'n A,
- Resuspend 100mm plate in 6ml cryogenic media (90% serum, 10%DMSO) and aliquot into 3- 2ml cryogenic vials
- Freeze at –150^oC or liquid nitrogen
- Use later by thawing a vial into one 100mm dish with complete medium

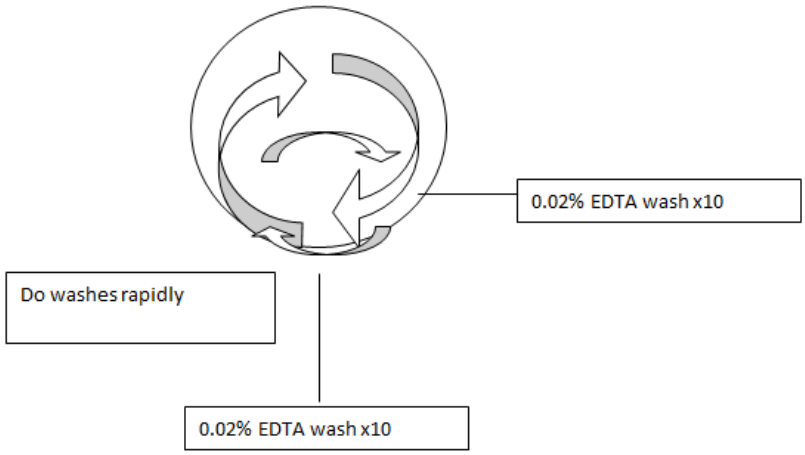
Note: The 3T3 cells must be used as feeder layers or frozen down within 2 days of being treated with mitomycin C

Splitting the feeder layer/epithelial plate:

6.) Once the epithelial cells on the plate are 70-75% confluent, aspirate off medium and wash once with pre-warmed Sol'n A (cells that are too confluent initiate differentiation).

7.) Aspirate off Sol'n A and wash with pre-warmed 0.02% EDTA (0.68mM) in 1x PBS. This wash should be performed fairly rapidly and is done to remove the Swiss 3T3 cells, but leave behind the epithelial cells. The EDTA wash is done ten times by pipetting 10-12mLs directly onto the cells on the outer perimeter of the plate, while rotating the plate. Then using this same EDTA again, do the same for the inner half of the plate cells.

8.) Diagram of 0.02% EDTA in 1x PBS washes:



9.) Aspirate off the EDTA from the previous washes, and repeat the 0.02% EDTA washes for the inner and outer halves of the plate. This makes a total of 40 washes.

This EDTA wash protocol is for human keratinocytes, other cells may be less strongly adherent and the number of washes may have to be modified.

10.) Aspirate off the EDTA and wash the plate with pre-warmed Sol'n A x3 still keeping the plate tilted. On the last Sol'n A wash look at the cells under the microscope and verify that all of the Swiss 3T3 are washed away, if they are not then more EDTA washes should be performed.

11.) Trypsinize the epithelial cells using their normal protocol (for keratinocytes trypsin 2 minutes RT, aspirate off the trysin and then incubate for 5 min at 37^{o}C, tap plate, resuspend in DMEM/F12). Epithelial cells should be plated back onto mitomycin C treated feeder layers in low density (250K for a 100mm plate).

Cell Synchronization

Synchronization literally means to make two or more things happen exactly simultaneously. For instance, two or more watches can be synchronized to show exactly the same time. The cells at different stages of the cell cycle in a culture can be synchronized so that the cells will be at the same phase. Cell synchrony is required to study the progression of cells through cell cycle. Several laboratory techniques have been developed to achieve cell synchronization.

They are broadly categorized into two groups:

1. Physical fractionation for cell separation.

2. Chemical blockade for cell separation.

Cell Separation by Physical Means:

Physical fractionation or cell separation techniques, based on the following characteristics are in use:

a. Cell density.
b. Cell size.
c. Affinity of antibodies on cell surface epitopes.
d. Light scatter or fluorescent emission by labeled cells.

The two commonly used techniques namely centrifugal elutriation and fluorescence-activated cell separation are briefly described hereunder.

Centrifugal elutriation:

The physical characteristics—cell size and sedimentation velocity are operative in the technique of centrifugal elutriation. Centrifugal elutriator (from Beckman) is an advanced device for increasing the sedimentation rate so that the yield and resolution of cells is better. The cell separation is carried out in a specially designed centrifuge and rotor (Fig. 2.4). The cells in the medium are pumped into the separating chamber while the rotor is turning.

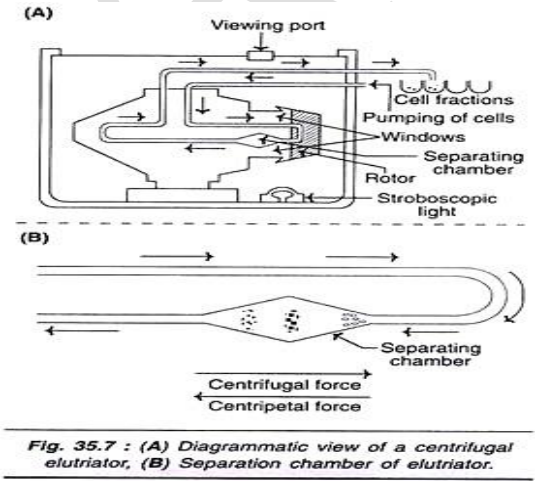


Fig. 35.7 : (A) Diagrammatic view of a centrifugal elutriator, (B) Separation chamber of elutriator.

Fig 2.4: specially designed centrifuge with rotor

Due to centrifugal force, the cell will be pushed to the edges. As the medium is then pumped through the chamber in such a way that the centripetal flow is equal to the sedimentation rate of cells. Due to differences in the cells (size, density, cell surface configuration), the cells tend to sediment at different rates, and reach equilibrium at different positions in the chamber.

The entire operation in the elutriator can be viewed through the port, as the chamber is illuminated by stroboscopic light. At the equilibrium the flow rate can be increased and the cells can be pumped out, and separated in collecting vessels in different fractions. It is possible to carry out separation of cells in a complete medium, so that the cells can be directly cultured after separation.

Fluorescence-activated cell sorting:

Fluorescence-activated cell sorting is a technique for sorting out the cells based on the differences that can be detected by light scatter (e.g. cell size) or fluorescence emission (by pretreated DNA, RNA, proteins, antigens). The procedure involves passing of a single stream of cells through a laser beam so that the scattered light from the cells can be detected and recorded. When the cells are pretreated with a fluorescent stain (e.g. chromomycin A for DNA), the fluorescent emission excited by the laser can be detected.

There are two instruments in use based on the principle of fluorescent-activated cell sorting:

1. Flow cytometer:

This instrument is capable of sorting out cells (from a population) in different phases of the cell cycle based on the measurements of a combination of cell size and DNA fluorescence.

2. Fluorescent-activated cell sorter (FACS):

In this instrument, the emission signals from the cells are measured, and the cells sorted out into collection tubes.

Comparison between physical methods:

For separation of a large number of cells, centrifugal elutriator is preferred. On the other hand, fluorescent-activated cell sorting is mostly used to obtain high grade pure fractions of cells from small quantities of cells.

Cell Separation by Chemical Blockade:

The cells can be separated by blocking metabolic reactions. Two types of metabolic blockades are in use — inhibition of DNA synthesis and nutritional deprivation.

Inhibition of DNA synthesis:

During the S phase of cell cycle, DNA synthesis can be inhibited by using inhibitors such as thymidine, aminopterine, hydroxyurea and cytosine arabinoside. The effects of these inhibitors are variable. The cell cycle is predominantly blocked in S phase that results in viable cells.

Nutritional deprivation:

Elimination of serum or isoleucine from the culture medium for about 24 hours results in the accumulation of cells at G_1 phase. This effect of nutritional deprivation can be restored by their addition by which time the cell synchrony occurs.

Some Highlights of Cell Synchronization:

a. Cell separation by physical methods is more effective than chemical procedures.

b. Chemical blockade is often toxic to the cells.

c. Transformed cells cannot be synchronized by nutritional deprivation.

d. A high degree of cell synchrony (>80%) can be obtained in the first cycle, and in the second cycle it would be <60%. The cell distribution may occur randomly in the third cycle.

How to perform cell synchronization in specific cell cycle phases

The cell cycle has been very well documented over the years because of its dysregulation in diseases such as cancer. Many different processes contribute to cell growth and replication, which is ultimately controlled by a series of tightly controlled cell cycle phases. For some areas of research, especially within drug discovery and cancer research, cell synchronization in a particular cell cycle phase can help to determine at which point, if any, their sensitivity to a drug is heightened. Alternatively, you might want to study the activity or properties of a particular cyclin or cyclin-dependent kinase. These experiments may be challenging when only a small fraction of your cultured cells are in the desired phase.

Briefly, the cell cycle exists as 3 main phases:

- G0/G1 phase – cell rest and recovery in preparation for subsequent rounds of cell division
- S Phase – DNA replication (interphase)
- G2/M phase – chromosome segregation and mitosis

Here, we present a small toolkit to help you on your way to cell synchronization to enrich the populations in various cell cycle phases. These methods are compatible with simple cell cycle flow cytometry analysis.

Cell Synchronization in G1

G1 Arrest by Serum Starvation

During G1, cells synthesize all of the molecules needed for a new round of cell division. The serum in growth media contains everything needed for this, including growth factors, which are important signals for cell division. Therefore, starving cells of serum can prevent them from dividing. You may need to carry out some optimization steps, but this is by far the cheapest way to arrest your cells! Bear in mind though that it doesn't necessarily work for all cell lines or cell types.

How to perform serum starvation:

1. Wash your cells in serum-free media
2. Re-suspend or seed them at the right concentration
3. Seed and incubate for anywhere between 24 and 72 hours.
4. Check for synchronization in G1 using DNA dyes in flow cytometry, as mentioned later in this article.

G1 Arrest by Double Thymidine Block

Excess thymidine is an effective inhibitor of DNA synthesis, thereby arresting cells either in G1 prior to DNA replication, or in S phase. This use of thymidine is known as a thymidine block. A double thymidine block ensures that any cells that were in mid or late S phase during the first block will be captured in late G1 or early S phase in the second block.

How to perform a double thymidine block:

1. Grow cells to or seed at the required concentration
2. Add thymidine (final concentration 2 mM) to your culture and incubate overnight

3. Wash cells and incubate for approx. 9 hours with deoxycytidine to release them from the block. In simple terms, deoxycytidine restores the imbalances in nucleotide pools that occur after exposure to excess thymidine
4. Repeat the thymidine incubation
5. Release again using deoxycytidine

G1 Arrest by Inhibition of CDKs

Specific pairs of cyclins and cyclin dependent kinases (CKDs) govern each phase of the cell cycle. Progression between phases is strictly controlled so that cells cannot grow and divide without passing stringent checkpoints.

We can take advantage of this by using a number of commercially available small molecule CDK inhibitors, which can effectively stop cell cycle progression, thus arresting or synchronizing cells in the desired phase.

CDK4/6 inhibitors are very effective at arresting your cells in G1 phase. Many of the marketed CDK inhibitors were actually developed by large pharmaceutical companies as anticancer drugs. You can take your pick from palbociclib (Pfizer), ribociclib (Novartis) or abemaciclib (Eli Lilly).

As long as you optimize inhibitor concentrations and the duration of treatment for your cell type, you can arrest your cells in G1 phase using this strategy. However, bear in mind that small molecule inhibitors can have off-target effects, and you should control for these in your experiments.

From personal experience, CDK inhibition works well across a wider panel of cancer cell lines than serum starvation does. Many cell lines are not amenable to serum starvation and will die in protest! Thymidine block is also an effective method but is more laborious and time consuming to perform.

Cell Synchronization in G2

G2 arrest by inhibition of microtubule formation

Before mitosis, where your cells divide into two daughter cells, chromosomal segregation occurs, ensuring the correct number and complement of chromosomes in daughter cells. The microtubules carry out segregation, by attaching to the kinetochore on the chromatid, thus physically pulling chromatids apart. Inhibition of microtubule formation is therefore a very efficient way of preventing mitosis, resulting in cell arrest at the G2 to M phase boundary. Colcemid, nocodazole, paclitaxel, vincristine and vinblastine are popular examples of microtubule inhibitors for synchronization in G2.

G2 Arrest by Inhibition of CDKs

CDK1 controls the G2 to M phase boundary. CDK1 inhibition by the small molecule inhibitor RO-3306 (Roche) can arrest cells in G2 phase. Again, success may depend on the cell line or cell type, but you can usually identify the right concentration and incubation time for successful G2 arrest with some optimization.

Many researchers prefer CDK inhibitors over microtubule inhibitors since they are generally less cytotoxic. This means your cells will still be alive after treatment, which is quite useful if you plan on further experimentation!

Synchronization in S Phase

During S phase, DNA synthesis occurs in preparation for subsequent rounds of cell division. A small molecule called 2[[3-(2,3-dichlorophenoxy)propyl] amino]ethanol (2,3-DCPE) was described as a potent inducer of apoptosis. This molecule works by activating certain caspases and reducing levels of the Bcl-XL protein (2). Through an unknown mechanism, this molecule

works very well at arresting your cells in S phase (3). This is a case of who cares how it works, as long as it works!

How to Confirm Cell Synchronization

As well as optimizing your synchronization protocol, is it also important to be able to show cell arrest in the desired phase. Microscopy is one interesting way to actually see what is happening inside your cells.

Alternatively, you can use flow cytometry to confirm synchronization by comparing your treated cells with an asynchronous control as follows:

1. Fix and permeabilize your cells in 70 % ethanol
2. Stain with 40 µg/ml propidium iodide, and include 25 µg/ml of RNase (to degrade RNA and ensure that you stain DNA only).
3. Run your samples on the flow cytometer and voila!

Cell synchronization can be a tricky business because you are trying to stop the cells from what they naturally do: divide!

Cryopreservation

Cryopreservation is the process of freezing biological material at extreme temperatures; most common -196 °C/-321 °F in liquid nitrogen (N_2). At these low temperatures, all biological activity stops, including the biochemical reactions that lead to cell death and DNA degradation. This preservation method in theory makes it possible to store living cells as well as other biological material unchanged for centuries.

The challenge of cryopreservation is to help cells to survive both cooling to extreme temperatures and thawing back to physiological conditions. Intracellular ice formation in particular is a critical issue that has to be controlled to keep the cell membrane intact and the

cells alive. The crucial elements to prevent this are the freezing rate (degrees per minute) and the composition of the freezing medium used. The freezing medium generally consists of a diluter, (sometimes) a protein source, as well as a cryoprotectant compound. The choice of most suitable cryoprotectant will influence the preservation result and will be different between different cells and different species.

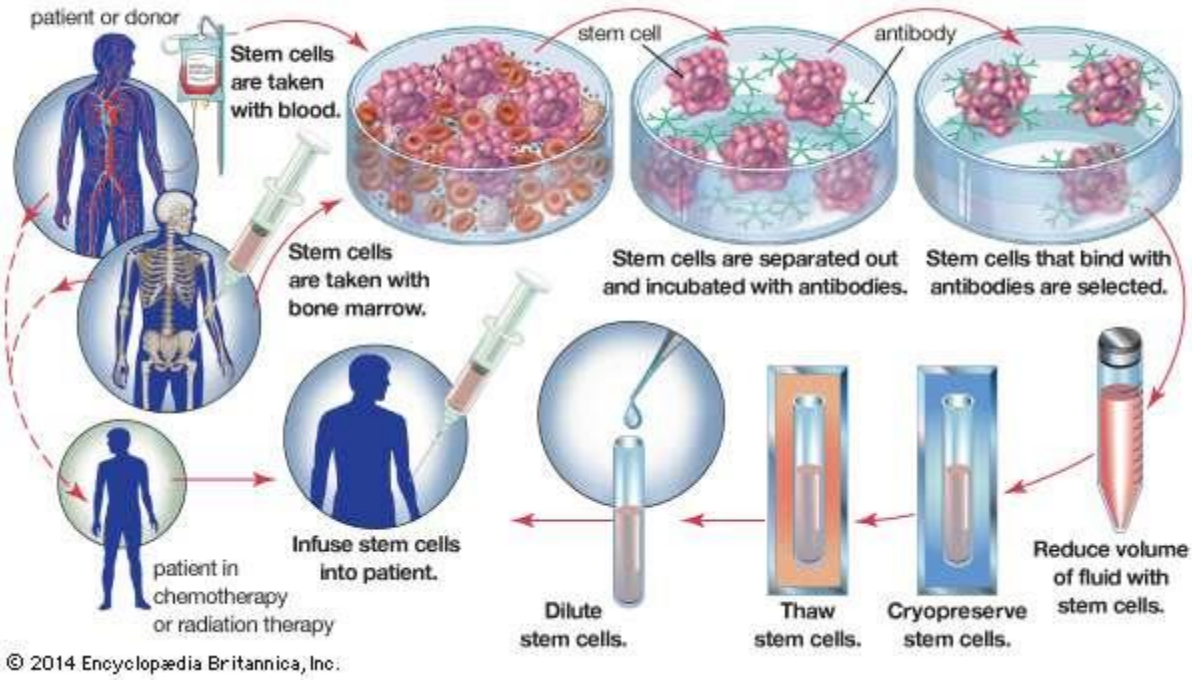
Cryopreservation technology is important in breeding programs to preserve desired genes, but also provides an opportunity to save endangered species.

Cryopreservation is based on the ability of certain small molecules to enter cells and prevent dehydration and formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the freezing process. Two common cryoprotective agents are dimethyl sulfoxide (DMSO) and glycerol. Glycerol is used primarily for cryoprotection of red blood cells, and DMSO is used for protection of most other cells and tissues. A sugar called trehalose, which occurs in organisms capable of surviving extreme dehydration, is used for freeze-drying methods of cryopreservation. Trehalose stabilizes cell membranes, and it is particularly useful for the preservation of sperm, stem cells, and blood cells.

Most systems of cellular cryopreservation use a controlled-rate freezer. This freezing system delivers liquid nitrogen into a closed chamber into which the cell suspension is placed. Careful monitoring of the rate of freezing helps to prevent rapid cellular dehydration and ice-crystal formation. In general, the cells are taken from room temperature to approximately −90 °C (−130 °F) in a controlled-rate freezer. The frozen cell suspension is then transferred into a liquid-nitrogen freezer maintained at extremely cold temperatures with nitrogen in either the vapour or the liquid phase. Cryopreservation based on freeze-drying does not require use of liquid-nitrogen freezers.

An important application of cryopreservation is in the freezing and storage of hematopoietic stem cells, which are found in the bone marrow and peripheral blood. In autologous bone-

marrow rescue, hematopoietic stem cells are collected from a patient's bone marrow prior to treatment with high-dose chemotherapy. Following treatment, the patient's cryopreserved cells are thawed and infused back into the body. This procedure is necessary, since high-dose chemotherapy is extremely toxic to the bone marrow. The ability to cryopreserve hematopoietic stem cells has greatly enhanced the outcome for the treatment of certain lymphomas and solid tumour malignancies. In the case of patients with leukemia, their blood cells are cancerous and cannot be used for autologous bone-marrow rescue. As a result, these patients rely on cryopreserved blood collected from the umbilical cords of newborn infants or on cryopreserved hematopoietic stem cells obtained from donors. Since the late 1990s it has been recognized that hematopoietic stem cells and mesenchymal stem cells (derived from embryonic connective tissue) are capable of differentiating into skeletal and cardiac muscle tissues, nerve tissue, and bone. Today there is intense interest in the growth of these cells in tissue culture systems, as well as in the cryopreservation of these cells for future therapy for a wide variety of disorders, including disorders of the nervous and muscle systems and diseases of the liver and heart.



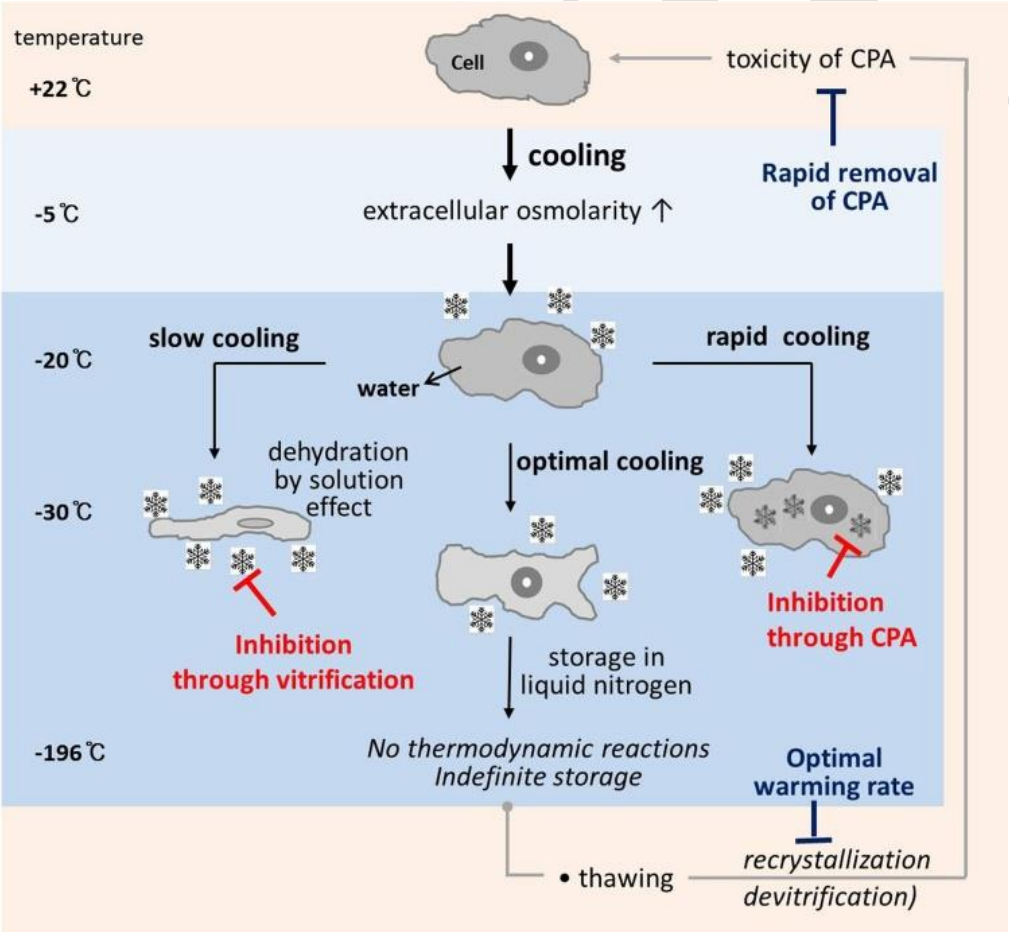
Bone marrow transplantationHigh doses of chemotherapy or radiation destroy not only cancer cells but also bone marrow, which is rich in blood-forming stem cells. In order to replace damaged marrow, stem cells are harvested from either the blood or the bone marrow of the cancer patient before therapy; cells also may be taken from a genetically compatible donor. In order to remove unwanted cells, such as tumour cells, from the sample, it is incubated with antibodies that bind only to stem cells. The fluid that contains the selected cells is reduced in volume and frozen until needed. The fluid is then thawed, diluted, and reinfused into the patient's body. Once in the bloodstream, the stem cells travel to the bone marrow, where they implant themselves and begin producing healthy cells.

Profound hypothermia, a form of mild cryopreservation used in human patients, has significant applications. A common use of induction of profound hypothermia is for complex cardiovascular surgical procedures. After the patient has been placed on complete cardiopulmonary bypass, using a heart-lung machine, the blood passes through a cooling chamber. Controlled cooling of the patient may reach extremely low temperatures of around 10–14 °C (50–57 °F). This amount of cooling effectively stops all cerebral activity and provides protection for all the vital organs. When this extreme cooling has been achieved, the heart-lung machine can be stopped, and the surgeon can correct very complex aortic and cardiac defects during circulatory arrest. During this time, no blood is circulating within the patient. After the surgery has been completed, the blood is gradually warmed in the same heat exchanger used for cooling. Gradual warming back to normal body temperatures results in resumption of normal brain and organ functions. This profound hypothermia, however, is far removed from freezing and long-term cryopreservation.

Cells can live more than a decade if properly frozen. In addition, certain tissues, such as parathyroid glands, veins, cardiac valves, and aortic tissue, can be successfully cryopreserved. Freezing is also used to store and maintain long-term viability of early human embryos, ova (eggs), and sperm. The freezing procedures used for these tissues are well established, and, in the

presence of cryoprotective agents, the tissues can be stored over long periods of time at temperatures of −14 °C (6.8 °F).

Research has shown that whole animals frozen in the absence of cryoprotective agents can yield viable cells containing intact DNA upon thawing. For example, nuclei of brain cells from whole mice stored at −20 °C (−4 °F) for more than 15 years have been used to generate lines of embryonic stem cells. These cells were subsequently used to produce mouse clones.



Physical events and cryoinjury of cells during freezing and thawing. Cryoinjuries are caused, at least in part, by the solution effect (leading to osmotic shock) and intracellular ice formation (leading to breakdown of intracellular structures).

Applications of cryopreservation

The applications of cryopreservation can be categorized into the following areas: (1) cryopreservation of cells or organs; (2) cryosurgery; (3) biochemistry and molecular biology; (4) food sciences; (5) ecology and plant physiology; and (6) many medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and *in vitro* fertilization (IVF). Some suggested advantages of cryopreservation include the possible banking of cells for human leukocyte antigen typing for organ transplantation, the allowance of sufficient time for transport of cells and tissues among different medical centers, and the provision of research sources for identifying unknown transmissible diseases or pathogens. Furthermore, the long-term storage of stem cells is still the initial step toward tissue engineering, which holds promise for the regeneration of soft tissue esthetic function and for the treatment of known diseases that have currently no therapy option.

Oocytes and embryos

The first case of embryo cryopreservation for fertility preservation took place in 1996, with the application of a natural IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer. Cryopreservation of mature oocytes is a proven technique for preserving the reproductive capacity. Results from a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle from 1986 to 2007 showed that there was no significant impact of the duration of storage on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles. Since oocytes are highly prone to chilling injury; cryopreservation of immature oocytes and ovarian tissue is a promising approach-with reports of live births-but the need for investigational improvements remain.

Sperm, semen, and testicular tissue

Germ cell depletion caused by chemical or physical toxicity, disease, or genetic predisposition can occur at any age. Fertility preservation is of great importance to guarantee the quality of life of patients facing chemo- and radiotherapy. Sperm and semen can be used almost indefinitely after proper cryopreservation. There are new trials for cryopreserving testicular tissues in the form of cell suspensions, tubular pieces, and entire gonads, but this technique is still premature. Overall, cryopreservation can be used as a first-line means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.

Stem cells

Adult stem cells are capable of differentiating into multiple types of specific cells and can be obtained from various locations other than bone marrow, including fat tissue, the periosteum, amniotic fluid, and umbilical cord blood. Stem cells can be subdivided into embryonic stem cells, mesenchymal stromal cells, and hematopoietic stem cells, all of which are considered as goldmines for potential application in regenerative medicine. Clearly, the fields of tissue engineering, gene therapy, regenerative medicine, and cell transplantation are largely dependent on the ability to preserve, store, and transport these stem cells without modification of their genetic and/or cellular contents.

Hepatocytes

Primarily isolated hepatocytes have found important applications in science and medicine over the past 40 years in a wide range of areas, including physiological studies, investigations on liver metabolism, organ preservation and drug detoxification, and experimental and clinical transplantation. In addition, there is currently increasing interest in the applications of liver

progenitor cells across a range of scientific areas, including both regenerative medicine and biotechnology, which raises the need for cryobanking.

Others

Although primary neuronal cells and cardiomyocytes are routinely used for neuroscience and cardiology research, a gold standard protocol for the preservation of these cells has not yet been developed. With the discovery of glucocorticoid-free immunosuppressive regimens, pancreatic islet transplantation may be considered as an alternative for the treatment of type 1 diabetes. For this reason, the development of islet cryopreservation methods has been ongoing, but results are still suboptimal, with a survival rate of less than 50%.

Limitations of cryopreservation

Although numerous usages of the cryopreservation technique exist, both in basic and clinical research, some limitations still exist. Cells metabolize almost nothing at low temperatures such as −196 °C (i.e., in liquid nitrogen), which has inevitable side effects, including a genetic drift toward biological variations of cell-associated changes in lipids and proteins that could result in the impairment of cellular activity and structure. If there were no limit to the amount of CPA that could be used, cells would be preserved perfectly. In conventional settings, however, CPAs themselves can be damaging to cells, especially when used in high concentrations. For example, there is a possibility that DMSO may alter chromosome stability, which can lead to a risk of tumor formation. Apart from endogenous changes in cells, the possible infection or contamination with cells such as tumorous ones should be prevented.

Types of Cells

Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics. There are three basic morphologies:

1. Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape.

2. Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape.

3. Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures. It is important to remember that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies. Using cell fusion techniques, it is also possible to obtain **hybrid cells** by fusing cells from two different parents. These may exhibit characteristics of either parent or both parents. This technique was used in 1975 to create cells capable of producing custom tailored monoclonal antibodies. These hybrid cells (called **Hybridomas**) are formed by fusing two different but related cells. The first is a spleen-derived lymphocyte that is capable of producing the desired antibody. The second is a rapidly dividing myeloma cell (a type of cancer cell) that has the machinery for making antibodies but is not programmed to produce any antibody. The resulting hybridomas can produce large quantities of the desired antibody.

These antibodies, called **Monoclonal Antibodies** due to their purity, have many important clinical, diagnostic, and industrial applications.

Functional Characteristics The characteristics of cultured cells result from both their origin (liver, heart, etc.) and how well they adapt to the culture conditions. Biochemical markers can be used to determine if cells are still carrying on specialized functions that they performed in vivo (e.g., liver cells secreting albumin). Morphological or ultra-structural markers can also be examined (e.g., beating heart cells). Frequently, these characteristics are either lost or changed as a result of being placed in an artificial environment. Some cell lines will eventually stop dividing and show signs of aging. These lines are called **Finite**. Other lines are, or become **immortal**; these can continue to divide **indefinitely** and are called **Continuous** cell lines. When a "normal" finite cell line becomes immortal, it has undergone a fundamental irreversible change or "transformation". This can occur spontaneously or be brought about intentionally using drugs,

radiation or viruses. **Transformed Cells** are usually easier and faster growing, may often have extra or abnormal chromosomes and frequently can be grown in suspension

What is Cell Culture Used For? Cell culture has become one of the major tools used in cell and molecular biology. Some of the important areas where cell culture is currently playing a major role are:

1- Model Systems Cell cultures provide a good model system for studying

- basic cell biology and biochemistry,
- the interactions between disease-causing agents and cells,
- the effects of drugs on cells,
- the process and triggers for aging, and
- nutritional studies

2- Toxicity Testing

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

3- Cancer Research Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells. Thus, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

4- Virology One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms.

5- Cell-Based Manufacturing While cultured cells can be used to produce many important products, three areas are generating the most interest. **The first** is the large-scale production of viruses for use in vaccine production. These include vaccines for polio, rabies, chicken pox,

hepatitis B and measles. **Second**, is the large-scale production of cells that have been genetically engineered to produce proteins that have medicinal or commercial value. These include monoclonal antibodies, insulin, hormones, etc. **Third**, is the use of cells as replacement tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product.

6- Genetic Counseling Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

7- Genetic Engineering The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of these genes (new proteins). These techniques can also be used to produce these new proteins in large quantity in cultured cells for further study.

8- Gene Therapy The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect"" the patient with the virus in the hope that the missing gene will then be expressed in the patient"s cells.

Tissue typing

Tissue typing is a group of procedures that determines the type of histocompatibility antigens on a person's cells or tissues. This procedure is typically used prior to transplantation of tissues or organs.

Purpose

Tissue typing is done prior to transplantation to ensure as close a match as possible between the donor and the recipient. If the histocompatibility antigens do not match well, there is a much greater chance that the recipient will reject the donated tissue.

Histocompatibility antigens are molecules on the surface of all cells in the body. The specific types of histocompatibility antigens present on a person's cells determine their identity and distinguish each person. They are a "fingerprint."

Each person has a unique set of histocompatibility antigens. If the antigens on tissue or organs from a donor do not match that of the recipient, a rejection response can occur. The recipient's immune system will detect the difference between the two sets of antigen and start a rejection response to kill the donated tissue. Except in the case of identical twins, no two people are identical in terms of their histocompatibility antigen types. However, the closer two tissues come to matching, the more likely the recipient will accept the donated tissue or organ.

Human Lymphocyte Antigens (HLA) is the name given to the most commonly used histocompatibility antigens. The antigens can be grouped into two classes: class I antigens are found on almost all cells, and class II antigens are normally found only on B lymphocytes, macrophages, monocytes, dendritic cells, and endothelial cells.

Description

Generally, typing is performed on blood cells because they are an easy sample to obtain. Blood is withdrawn from a vein in the forearm, and the cells are separated. There are a number of different techniques used to identify the antigens on the cells. Typically, specific antibodies react with the cells. Each antibody preparation is specific for one histocompatibility antigen. If the antigen is present, the antibody will bind to it. Laboratory instruments are used to detect antibody binding to the cells. Class II antigens are determined by the mixed lymphocyte reaction (MLR) or by a PCR. In the mixed lymphocyte reaction, lymphocyte replication occurs if there is a

mismatch, and is detected by a specific assay. The PCR test is a new DNA-based test that can detect the presence or absence of antigens by determining whether cells have the genes for the antigens.

One type of transplant does not require tissue typing. In the case of corneal transplants, tissue typing is not needed because cornea do not have their own blood supply. This greatly reduces the chance that immune cells will come in contact with the cornea and recognize it as foreign. For this reason, corneas can be transplant from any person, and there is little chance of rejection.

Normal results

Because each person has their own histocompatibility antigen "fingerprint," there is no true normal result. Each fingerprint is unique.

Characteristics of Cultured Cells:

Some of the important distinguishing properties of cultured cells are given below:

1. Cells which do not normally proliferate in vivo can be grown and proliferated in cultures.
2. Cell to cell interactions in the cultured cells are very much low.
3. The three dimensional architecture of the in vivo cells is not found in cultured cells.
4. The hormonal and nutritional influence on the cultured cells differs from that on the in vivo cells.
5. Cultured cells cannot perform differentiated and specialized functions.
6. The environment of the cultured cells favours proliferation and spreading of unspecialized cells.

Environmental influence on cultured cells:

The environmental factors strongly influence the cells in culture. The major routes through which environmental influence occurs are listed:

i. The nature of the substrate or phase in which cells grow. For monolayer cultures, the substrate is a solid (e.g. plastic) while for suspension cultures, it is a liquid.

ii. The composition of the medium used for culture nutrients and physicochemical properties.

iii. Addition of hormones and growth factors.

iv. The composition of the gas phase.

v. The temperature of culture incubation.

The biological and other aspects of cultured cells with special reference to the following parameters are briefly described:

1. Cell adhesion.

2. Cell proliferation.

3. Cell differentiation.

4. Metabolism of cultured cells.

5. Initiation of cell culture.

6. Evolution and development of cell lines.

Cell Adhesion:

Most of the cells obtained from solid tissues grow as adherent monolayers in cultures. The cells, derived from tissue aggregation or subculture, attach to the substrate and then start proliferating. In the early days of culture techniques, slightly negatively charged glasses were used as substrates. In recent years, plastics such as polystyrene, after treatment with electric ion discharge, are in use.

The cell adhesion occurs through cell surface receptors for the molecules in the extracellular matrix. It appears that the cells secrete matrix proteins which spread on the substrate. Then the cells bind to matrix through receptors. It is a common observation that the substrates (glass or plastic) with previous cell culture are conditioned to provide better surface area for adhesion.

Cell adhesion molecules:

Three groups of proteins collectively referred to as cell adhesion molecules (CAMs) are involved in the cell-cell adhesion and cell-substrate adhesion.

Cell-cell adhesion molecules:

These proteins are primarily involved in cell-to-cell interaction between the homologous cells. CAMs are of two types — calcium-dependent ones (cadherin's) and calcium-independent CAMs.

Integrin's:

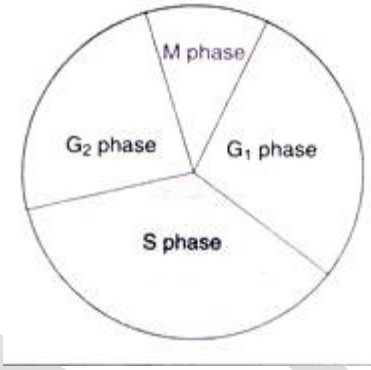
These molecules mediate the cell substrate interactions. Integrin's possess receptors for matrix molecules such as fibronectin and collagen.

Proteoglycans:

These are low affinity trans membrane receptors. Proteoglycans can bind to matrix collagen and growth factors. Cell adhesion molecules are attached to the cytoskeletons of the cultured cells.

Cell Proliferation:

Proliferation of cultured cells occurs through the cell cycle, which has four distinct phases (Fig. 2.5)



M phase:

In this phase (M = mitosis), the two chromatids, which constitute the chromosomes, segregate to daughter cells.

G_1 phase:

This gap 1 phase is highly susceptible to various control processes that determine whether cell should proceed towards DNA synthesis, re-enter the cycle or take the course towards differentiation.

S phase:

This phase is characterized by DNA synthesis wherein DNA replication occurs.

G_2 phase:

This is gap 2 phase that prepares the cell for reentry into mitosis. The integrity of the DNA, its repair or entry into apoptosis (programmed cell death) if repair is not possible is determined by two check points-at the beginning of DNA synthesis and in G_2 phase.

Control of cell proliferation:

For the cells in culture, the environmental signals regulate the cell cycle and thereby the cell proliferation. Low density of the cells in a medium coupled with the presence certain growth factors (e.g. epidermal growth factor, platelet-derived growth factor) allows the cells to enter the cell cycle.

On the other hand, high cell density and crowding of cells inhibits the cell cycle and thereby proliferation. Besides the influence of the environmental factors, certain intracellular factors also regulate the cell cycle. For instance, cyclins promote while p53 and Rb gene products inhibit cell cycle.

Cell Differentiation:

The various cell culture conditions favour maximum cell proliferation and propagation of cell lines.

Among the factors that promote cell proliferation, the following are important:

i. Low cell density

ii. Low Ca^{2+} concentration

iii. Presence of growth factors

For the process of cell differentiation to occur, the proliferation of cells has to be severely limited or completely abolished.

Cell differentiation can be promoted (or induced) by the following factors:

i. High cell density.

ii. High Ca^{2+} concentration.

iii. Presence of differentiation inducers (e.g. hydrocortisone, nerve growth factor).

As is evident from the above, different and almost opposing conditions are required for cell proliferation, and for cell differentiation. Therefore if cell differentiation is required two distinct sets of conditions are necessary.

1. To optimize cell proliferation.

2. To optimize cell differentiation.

Maintenance of differentiation:

It is now recognized that the cells retain their native and original functions for long when their three dimensional structures are retained. This is possible with organ cultures. However, organ cultures cannot be propagated.

In recent years, some workers are trying to create three dimensional structures by per-fusing monolayer cultures. Further, in vitro culturing of cells on or in special matrices (e.g. cellulose, collagen gel, matrix of glycoproteins) also results in cells with three dimensional structures.

Dedifferentiation:

Dedifferentiation refers to the irreversible loss of specialized properties of cells when they are cultured in vitro. This happens when the differentiated in vitro cells lose their properties (Fig. 2.6). In the in vivo situation, a small group of stem cells give rise to progenitor cells that are capable of producing differentiated cell pool (Fig. 2.6A).

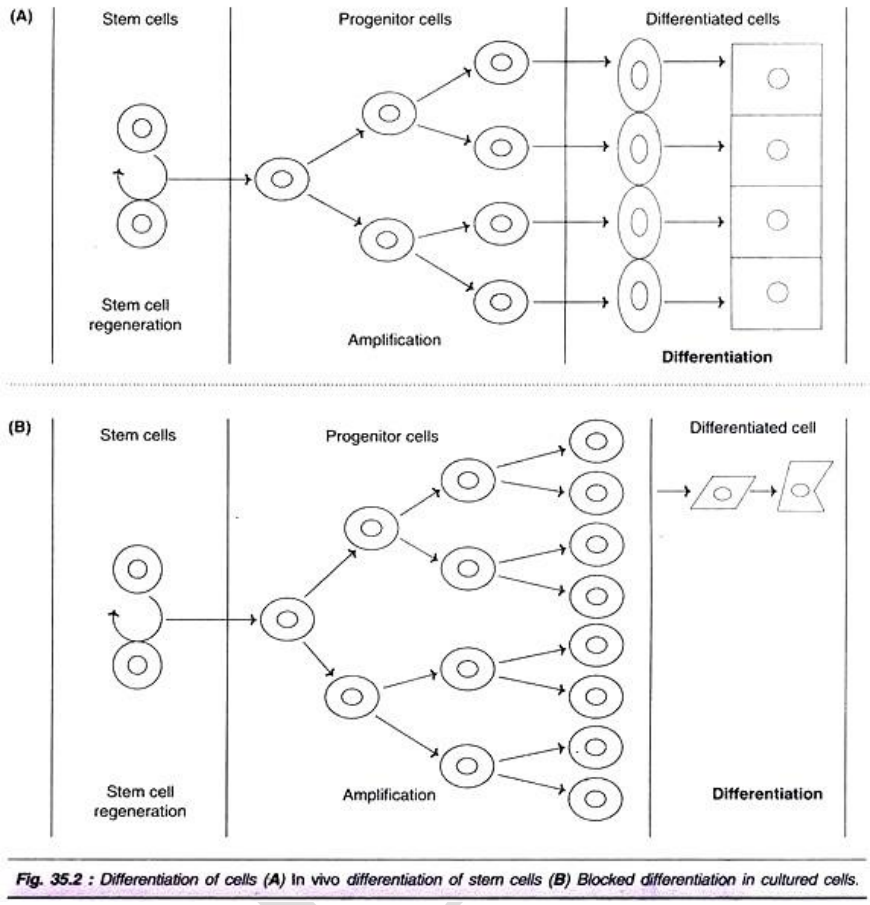


Fig. 35.2 : Differentiation of cells (A) In vivo differentiation of stem cells (B) Blocked differentiation in cultured cells.

On the other hand, in the in vitro culture system, progenitor cells are predominantly produced which go on proliferating. Very few of the newly formed cells can form differentiated cells (Fig. 2.6B). The net result is a blocked differentiation. Dedifferentiation implies an irreversible loss of specialized properties of the cells. On the other hand, de-adaptation refers to the re-induction of specialized properties of the cells by creating appropriate conditions.

Metabolism of Cultured Cells:

The metabolism of mammalian cultured cells with special reference to energy aspects is depicted in Fig. 2.7. The cultured cells can use glucose or glutamine as the source of energy. These two compounds also generate important anabolic precursors.

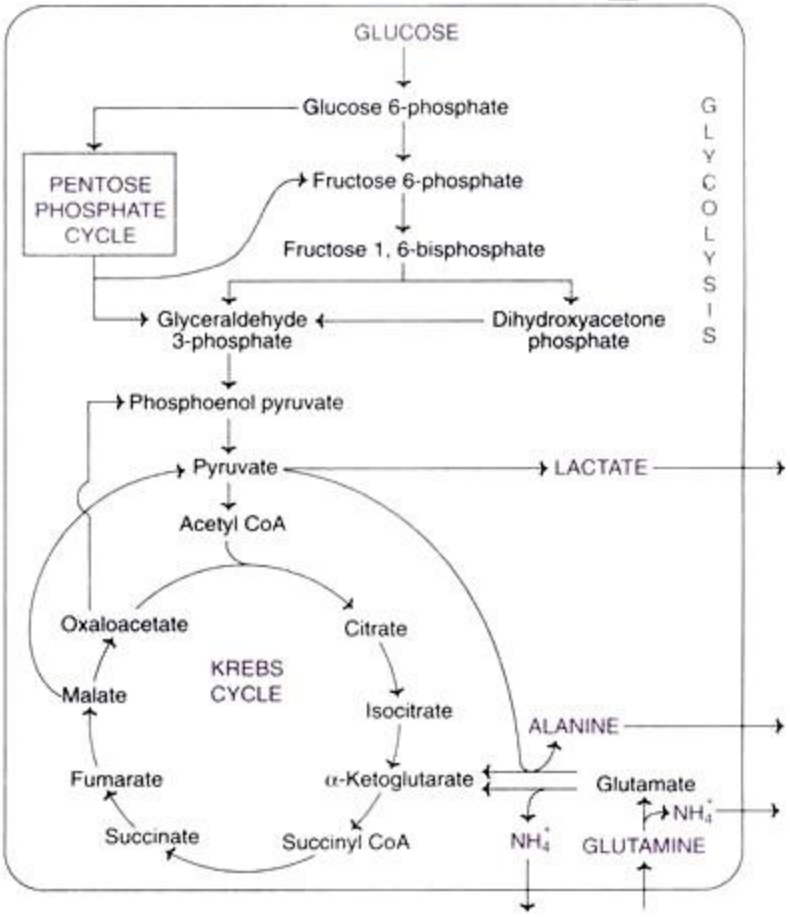


Fig. 35.3 : An outline of the glucose and glutamine metabolism in cultured mammalian cells.

As glucose gets degraded by glycolysis, lactate is mainly produced. This is because oxygen is in limited supply in the normal culture conditions (i.e. atmospheric oxygen and a submerged culture) creating an anaerobic situation. Lactate, secreted into the medium, accumulates.

Some amount of pyruvate produced in glycolysis gets oxidized through Krebs cycle. A small fraction of glucose (4-9%) enters pentose phosphate pathway to supply ribose 5-phosphate and reducing equivalents (NADPH) for biosynthetic pathways e.g. synthesis of nucleotides.

Glutamine is an important source of energy for the cultured cells. By the action of the enzyme glutaminase, glutamine undergoes deamination to produce glutamate and ammonium ions. Glutamate, on transamination (or oxidative deamination) forms a-ketoglutarate which enters the Krebs cycle.

Pyruvate predominantly participates in transamination reaction to produce alanine, which is easily excreted into the medium. In the rapidly growing cultured cells, transamination reaction is a dominant route of glutamine metabolism.

Deamination of glutamine releases free ammonium ions, which are toxic to the cultured cells, limiting their growth. In recent years, dipeptides glutamyl-alanine or glutamyl-glycine are being used to minimize the production of ammonia. Further, these dipeptides are more stable in the medium.

As already stated, α-ketoglutarate obtained from glutamine (via glutamate) enters the Krebs cycle and gets oxidized to carbon dioxide and water. For proper operation of Kerbs cycle, balancing of the intermediates of the cycle is required.

Two metabolites of Kerbs cycle namely malate and oxaloacetate leave the cycle and get converted respectively to pyruvate and phosphoenol pyruvate. The latter two compounds can reenter the Krebs cycle in the form of acetyl CoA. Thus, the continuity of Kerbs cycle is maintained. Glucose as well as glutamine gets metabolised by the cultured cells to supply energy in the form of ATP.

Initiation of Cell Culture:

The cell culture can be initiated by the cells derived from a tissue through enzymatic or mechanical treatments. Primary culture is a selective process that finally results in a relatively uniform cell line. The selection occurs by virtue of the capacity of the cells to survive as monolayer cultures (by adhering to substrates) or as suspension cultures.

Among the cultured cells, some cells can grow and proliferate while some are unable to survive under the culture environment. The cells continue to grow in monolayer cultures, till the availability of the substrate is occupied.

The term confluence is used when the cultured cells make close contact with one another by fully utilizing the available growth area. For certain cells, which are sensitive to growth limitation due to density, the cells stop growing once confluence is reached. However, the transformed cells are insensitive to confluence and continue to overgrow.

When the culture becomes confluent, the cells possess the following characters:

1. The closest morphological resemblance to the tissue of origin (i.e. parent tissue).

2. The expression of specialized functions of the cells comparable to that of the native cells.

Evolution and Development of Cell Lines:

The primary culture grown after the first subculture is referred to as cell line. A given cell line may be propagated by further sub culturing. As the subcultures are repeated, the most rapidly proliferating cells dominate while the non- proliferating or slowly proliferating cells will get diluted, and consequently disappear.

Senescence:

The genetically determined event of cell divisions for a limited number of times (i.e. population doublings), followed by their death in a normal tissue is referred to as senescence. However, germ cells and transformed cells are capable of continuously proliferating. In the in vitro culture, transformed cells can give rise to continuous cell lines.

The evolution of a continuous cell line is depicted in Fig. 2.8. The cumulative cell number in a culture is represented on Y-axis on a log scale, while the X-axis represents the time in weeks. The time for development of a continuous cell line is variable. For instance, for human diploid fibroblasts, the continuous cell line arises at about 14 weeks while the senescence may occur between 10 to 20 weeks; usually after 30 and 60 cell doublings.

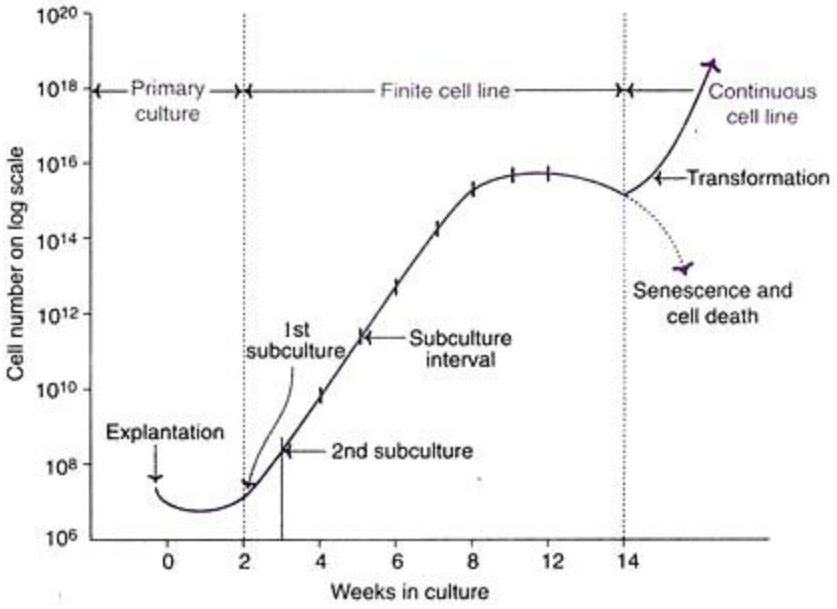


Fig. 35.4 : Diagrammatic representation of evolution of a cell line.

Development of continuous cell lines:

Certain alterations in the culture collectively referred to as transformation, can give rise to continuous cell lines. Transformation may be spontaneously occurring, chemically or virally-

induced. Transformation basically involves an alteration in growth characteristics such as loss of contact inhibition, density limitation of growth and anchorage independence. The term immortalization is frequently used for the acquisition of infinite life span to cultured cells.

Genetic variations:

The ability of the cells to grow continuously in cell lines represents genetic variation in the cells. Most often, the deletion or mutation of the p^{53} gene is responsible for continuous proliferation of cells. In the normal cells, the normal p^{53} gene is responsible for the arrest of cell cycle. Most of the continuous cell lines are aneuploid, possessing chromosome number between diploid and tetraploid value.

Normal cells and continuous cell lines:

A great majority of normal cells are not capable of giving rise to continuous cell lines. For instance, normal human fibroblasts go on proliferating for about 50 generations, and then stop dividing. However, they remain viable for about 18 months. And throughout their life span, fibroblasts remain euploid. Chick fibroblasts also behave in a similar fashion. Epidermal cells and lymphoblastic cells are capable of forming continuous cell lines.

Characterization of Cultured Cells:

Characterization of cultured cells or cell lines is important for dissemination of cell lines through cell banks, and to establish contacts between research laboratories and commercial companies.

Characterization of cell lines with special reference to the following aspects is generally done:

1. Morphology of cells

2. Species of origin.

3. Tissue of origin.

4. Whether cell line is transformed or not.

5. Identification of specific cell lines.

Morphology of Cells:

A simple and direct identification of the cultured cells can be done by observing their morphological characteristics. However, the morphology has to be viewed with caution since it is largely dependent on the culture environment. For instance, the epithelial cells growing at the center (of the culture) are regular polygonal with clearly defined edges, while those growing at the periphery are irregular and distended (swollen).

The composition of the culture medium and the alterations in the substrate also influence the cellular morphology. In a tissue culture laboratory, the terms fibroblastic and epithelial are commonly used to describe the appearance of the cells rather than their origin.

Fibroblastic cells:

For these cells, the length is usually more than twice of their width. Fibroblastic cells are bipolar or multipolar in nature.

Epithelial cells:

These cells are polygonal in nature with regular dimensions and usually grow in monolayers. The terms fibroblastoid (fibroblast-like) and epitheloid (epithelial-like) are in use for the cells that do not possess specific characters to identify as fibroblastic or epithelial cells.

Species of Origin of Cells:

The identification of the species of cell lines can be done by:

a. Chromosomal analysis.

b. Electrophoresis of isoenzymes.

c. A combination of both these methods.

In recent years, chromosomal identification is being done by employing molecular probes.

Identification of Tissue of Origin:

The identification of cell lines with regard to tissue of origin is carried out with reference to the following two characteristics:

1. The lineage to which the cells belong.

2. The status of the cells i.e. stems cells, precursor cells.

Tissue markers for cell line identification:

Some of the important tissue or lineage markers for cell line identification are briefly described.

Differentiated products as cell markers:

The cultured cells, on complete expression, are capable of producing differentiation markers, which serve as cell markers for identification.

Some examples are given below:

a. Albumin for hepatocytes.

b. Melanin for melanocytes

c. Hemoglobin for erythroid cells

d. Myosin (or tropomyosin) for muscle cells.

Enzymes as tissue markers:

The identification of enzymes in culture cells can be made with reference to the following characters:

a. Constitutive enzymes.

b. Inducible enzymes.

c. Isoenzymes.

The commonly used enzyme markers for cell line identification are given in Table 35.1.

TABLE 35.1 Some common enzyme markers for cell line identification

Enzyme	*Cell type*
Tyrosine aminotransferase	Hepatocytes
Tysosinase	Melanocytes
Glutamyl synthase	Brain (astroglia)
Creatine kinase (isoenzyme MM)	Muscle cells
Creatine kinase (isoenzyme BB)	Neurons, neuroendocrine cells
Non-specific esterase	Macrophages
DOPA-decarboxylase	Neurons
Alkaline phosphatase	Enterocytes, type II pneumocytes
Angiotensin–converting enzyme	Endothelium
Sucrase	Enterocytes
Neuron-specific esterase	Neurons

Tyrosine aminotransferase is specific for hepatocytes, while tyrosinase is for melanocytes. Creatine kinase (MM) in serum serves as a marker for muscle cells, while creatine kinase (BB) is used for the detection of neurons and neuroendocrine cells.

Filament proteins as tissue markers:

The intermediate filament proteins are very widely used as tissue or lineage markers.

For example:

a. Astrocytes can be detected by glial fibrillary acidic protein (GFAP).

b. Muscle cells can be identified by desmin.

c. Epithelial and mesothelial cells by cytokeratin.

Cell surface antigens as tissue markers:

The antigens of the cultured cells are useful for the detection of tissue or cells of origin. In fact, many antibodies have been developed (commercial kits are available) for the identification cell lines (Table. 35.2). These antibodies are raised against cell surface antigens or other proteins.

TABLE 35.2 A selected list of antibodies used for the detection of cell types

Antibody	*Cell type*
Cytokeratin	Epithelium
Epithelial membrane antigen	Epithelium
Albumin	Hepatocytes
α-Lactalbumin	Breast epithelium
Carcinoembryonic antigen (CEA)	Colorectal and lung adenocarcinoma
Prostate specific antigen (PSA)	Prostatic epithelium
Intracellular cell adhesion molecule (I-CAM)	T-cells and endothelium
α-Fetoprotein	Fetal hepatocytes
Human chorionic gonadotropin (hCG)	Placental epithelium
Human growth hormone (hGH)	Anterior pituitary
Vimentin	Mesodermal cells
Integrins	All cells
Actin	All cells

The antibodies raised against secreted antigen a-fetoprotein serves as a marker for the identification of fetal hepatocytes. Antibodies of cell surface antigens namely integrin's can be used for the general detection of cell lines.

Transformed Cells:

Transformation is the phenomenon of the change in phenotype due to the acquirement of new genetic material. Transformation is associated with promotion of genetic instability.

The transformed and cultured cells exhibit alterations in many characters with reference to:

a. Growth rate

b. Mode of growth

c. Longevity

d. Tumorigenicity

e. Specialized product formation.

While characterizing the cell lines, it is necessary to consider the above characters to determine whether the cell line has originated from tumor cells or has undergone transformation in culture.

Identification of Specific Cell Lines:

There are many approaches in a culture laboratory to identify specific cell lines:

a. Chromosome analysis

b. DNA detection

c. RNA and protein analysis

d. Enzyme activities

e. Antigenic markers.

Chromosome analysis:

The species and sex from which the cell line is derived can be identified by chromosome analysis. Further, it is also possible to distinguish normal and malignant cells by the analysis of chromosomes. It may be noted that the normal cells contain more stable chromosomes. The important techniques employed with regard to chromosome analysis are briefly described.

Chromosome banding:

By this technique, it is possible to identify individual chromosome pairs when there is little morphological difference between them. Chromosome banding can be done by using Giemsa staining.

Chromosome count:

A direct count of chromosomes can be done per spread between 50-100 spreads. A camera Lucida attachment or a closed circuit television may be useful.

Chromosome karyotyping:

In this technique, the chromosomes are cut, sorted into sequence, and then pasted on to a sheet. The image can be recorded or scanned from the slide. Chromosome karyotyping is time consuming when compared to chromosome counting.

DNA detection:

The total quantity of DNA per normal cell is quite constant, and is characteristic to the species of origin, e.g. normal cell lines from human, chick and hamster fibroblasts. However, the DNA content varies in the normal cell lines of mouse, and also the cell lines obtained from cancerous tissues. Most of the transformed cells are aneuploid and heteroploid. DNA analysis is particularly useful for characterization of such cells. Analysis of DNA can be carried out by DNA hybridization and DNA fingerprinting.

DNA hybridization:

The popular Southern blotting technique can be used to detect unique DNA sequences. Specific molecular probes with radioisotope, fluorescent or luminescent labels can be used for this

purpose. The DNA from the desired cell lines is extracted, cut with restriction endonucleases, subjected to electrophoresis, blotted on to nitrocellulose, and then hybridized with a molecular (labeled) probe, or a set of probes. By this approach, specific sequences of DNA in the cell lines can be detected.

DNA fingerprinting:

There are certain regions in the DNA of a cell that are not transcribed. These regions, referred to as satellite DNA, have no known functions, and it is believed that they may provide reservoir for genetic evolution. Satellite DNA regions are considered as regions of hyper variability. These regions may be cut with specific restriction endonucleases, and detected by using cDNA probes.

By using electrophoresis and autoradiography, the patterns of satellite DNA variations can be detected. Such patterns referred to as DNA fingerprints are cell line specific. In recent years, the technique of DNA fingerprinting has become a very popular and a powerful tool to determine the origin of cell lines.

RNA and protein analysis:

The phenotype characteristics of a cell line can be detected by gene expression i.e. identification of RNAs and/or proteins. mRNAs can be identified by Northern blot technique while proteins can be detected by Western blot technique.

Enzyme activities:

Some of the in vivo enzyme activities are lost when the cells are cultured in vitro. For instance, arginase activity of the liver cells is lost within a few days of culturing. However, certain cell lines express specific enzymes that can be employed for their detection e.g. tyrosine aminotransferase for hepatocytes, glutamyl synthase activity for astroglia in brain. For more examples of enzymes useful in cell line detection, refer Table 35.1.

Isoenzymes:

The multiple forms of an enzyme catalysing the same reaction are referred to as isoenzymes or isozymes. Isoenzymes differ in many physical and chemical properties—structure, electrophoretic and immunological properties, K_m and V_{max} values.

The isoenzymes can be separated by analytical techniques such as electrophoresis and chromatography. Most frequently, electrophoresis by employing agarose, cellulose acetate, starch and polyacrylamide is used. The crude enzyme is applied at one point on the electrophoretic medium. As the isoenzymes migrate, they distribute in different bands, which can be detected by staining with suitable chromogenic substrates.

Isoenzymes are characteristic to the species or tissues. Isoenzymes of the following enzymes are commonly used for cell line detection:

a. Lactate dehydrogenase

b. Malate dehydrogenase

c. Glucose 6-phosphate dehydrogenase

d. Aspartate aminotransferase

e. Peptidase B.

Isoenzyme analysis is also useful for the detection of interspecies cross-contamination of cell lines. For instance, contamination of mouse cell line with hamster cell line can be identified by using peptidase B isoenzymes.

Antigenic markers:

Cell lines can be characterized by detection of antigenic markers through the use of antibodies. The antigenic markers may be located on the cell surface or secreted by the cells into the culture medium. Some of the antibodies in common use for the detection of different cell types are given in Table 35.2 (See p. 430).

Measurement of Growth Parameters of Cultured Cells:

Information on the growth state of a given culture is required to:

a. Design culture experiments.

b. Routine maintenance of culture.

c. Measurement of cell proliferation.

d. Know the time for subculture.

e. Determine the culture response to a particular stimulus or toxin.

Some of the commonly used terms in relation to the measurement of growth of cultured cells are explained.

Population doubling time (PDT):

The time interval for the cell population to double at the middle of the logarithmic (log) phase.

Cell cycle time or generation time:

The interval from one point in the cell division to the same point in the cycle, one division later. Thus cell cycle time is measured form one point in the cell cycle until the same point is reached again.

Confluence:

It denotes the culture stage wherein all the available substrate (growth area) is utilized, and the cells are in close contact with each other.

Contact inhibition:

Inhibition of cell motility and plasma membrane ruffling when the cells are in complete contact with other adjacent cells. This mostly occurs at confluence state, and results in the ceasation of the cell proliferation.

Cell density:

The number of cells per ml of the medium.

Saturation density:

The density of the cells (cells/ml^2, surface area) in the plateau phase.

Growth Cycle of Cultured Cells:

The growth cycle of cultured cells is conventionally represented by three phases — the lag phase, the log (exponential) phase and the plateau phase (Fig. 2.9). The properties of the cultured cells vary in the phases.

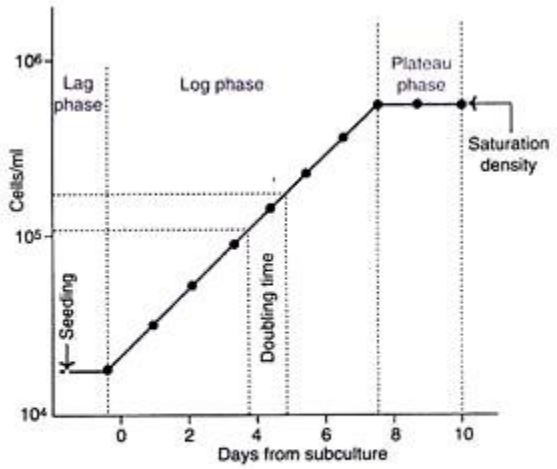


*Fig. 35.5 : Growth curve of cultured cells (**Note** : The cell concentration is expressed in semilog plot).*

The lag phase:

The lag phase represents a period of adaptation during which the cell forms the cell surface and extracellular matrix (lost during trypsinization), attaches to the substrate and spreads out. There is an increased synthesis of certain enzymes (e.g. DNA polymerase) and structural proteins, preparing the cells for proliferation.

The production of specialized products disappears which may not reappear until the cell proliferation ceases. The lag phase represents preparative stage of the cells for proliferation following subculture and reseeding.

The log phase:

The log phase is characterized by an exponential growth of cells, following the lag phase.

The duration of log phase depends on the cells with reference to:

a. Seeding density.

b. Growth rate.

c. Density after proliferation.

During the log phase, the cultured cells are in the most uniform and reproducible state with high viability. This is an ideal time for sampling. The log phase terminates after confluence is reached with an addition of one or two population doublings.

The plateau phase:

As the cells reach confluence, the growth rate is much reduced, and the proliferation of cultured cells almost stops.

This stage represents plateau or stationary phase, and is characterized by:

a. Low motility of cells.

b. Reduced ruffling of plasma membrane.

c. Cells occupying minimum surface area.

d. Contact inhibition.

e. Saturation density.

f. Depletion of nutrients and growth factors.

g. Reduced synthesis of structural proteins.

h. Increased formation of specialized products.

The majority of normal cultured cells that form monolayers stop growing as they reach confluence. Some of the cells however, with replenishment of medium continue to grow (at a

reduced rate) after confluence, forming multilayers of cells. The transformed cultured cells usually reach a higher cell density compared to the normal cells in the plateau phase (Fig. 2.9).

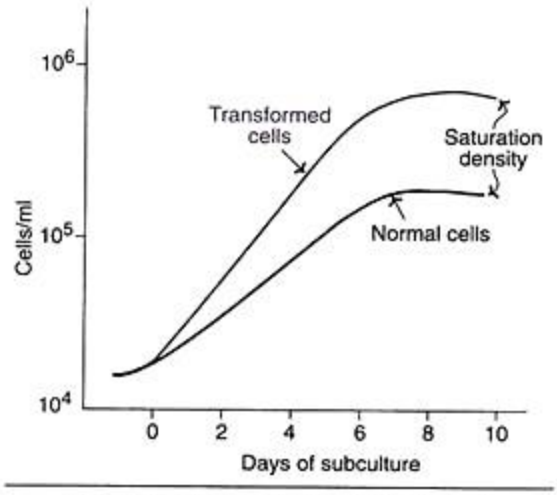


*Fig. 35.6 : Growth curves of transformed and normal cells (**Note** : The cell concentration is expressed in semilog plot).*

Plating Efficiency of Cultured Cells:

Plating efficiency, representing colony formation at low cell density, is a measure used for analyzing cell proliferation and survival.

When the cells, at low densities, are cultured in the form of single cell suspensions, they grow as discrete colonies. Plating efficiency is calculated as follows.

Plating efficiency = No. of colonies formed/No. of cells seeded × 100

The term cloning efficiency is used (instead of plating efficiency) when each colony grows from a single cell.

Seeding efficiency representing the survival of cells at higher densities is calculated as follows.

Seeding efficiency = No. of cells recovered/No. of cells seeded × 100

Measurement of cell death – apoptosis and its determination

Cellular Senescence and Apoptosis:

As the cells grow in culture, they become old due to aging, and they cannot proliferate any more. The end of the proliferative life span of cells is referred to as senescence.

Cellular Senescence:

The growth of the cells is usually measured as population doublings (PDs). The PDs refer to the number of times the cell population doubles in number during the period of culture and is calculated by the following formula.

Log_{10} (No. of cells harvested) – log_{10} (No. of cells seeded)/ $log10^2$

The phenomenon of senescence has been mostly studied with human fibroblast cultures. After 30-60 populations doublings, the culture is mainly composed of senescent fibroblasts. These senescent fibroblast are unable to divide in response to mitotic stimuli. It must be noted that the cells do not appear suddenly, but they gradually accumulate and increase in number during the life span of the culture.

The different parameters used for the measurement of cell growth in cultures are listed below:

a. Direct measure of cell number.

b. Determination of DNA/RNA content.

c. Estimation of protein/ATP concentration.

Measurement of Senescence:

The direct measurement of senescent cells is rather difficult.

Some of the indirect measures are:

a. Loss of metabolic activity

b. Lack of labeled precursor (^{3}H-thymidine) incorporation into DNA.

c. Certain histochemical techniques.

Senescence-associated β-galactosidase activity assay

There occurs an overexpression of the lysosomal enzyme β-galactosidase at senescence. This enzyme elevation is also associated with an increase in the cell size as the cell enters a permanent non-dividing state. The number of senescent cells in a culture can be measured by senescence-associated β-galactosidase (SA-β) assay.

The assay consists of the following stages:

1. Wash the cells and fix them using a fixative (e.g. para formaldehyde), and wash again.

2. Add the staining solution (X-gal powder in dimethylformamide dissolved in buffer) to the fixed cells and incubate.

3. The senescent cells display a dense blue colour which can be counted.

Apoptosis:

The process of programmed cell death (PCD) is referred to as apoptosis. The cell death may be initiated by a specific stimulus or as a result of several signals received from the external environment. Apoptosis occurs as a result of inherent cellular mechanisms, which finally lead to self-destruction. The cell activates a series of molecular events that cause an orderly degradation of the cellular constituents with minimal impact on the neighbouring tissues.

Reasons for in situ apoptosis:

1. For proper development:

The formation of fingers and toes of the fetus requires the removal of the tissues between them. This is usually carried out by apoptosis.

2. Destruction of cells that pose threat to the integrity of the organism:

Programmed cell death is needed to destroy and remove the cells that may otherwise damage the organisms.

Some examples are listed:

a. Cells with damaged DNA during the course of embryonic development. If they are not destroyed, they may result in birth defects.

b. Cells of the immune system, after their appropriate immune function, undergo apoptosis. This is needed to prevent autoimmune diseases e.g. rheumatoid arthritis.

c. Cells infected with viruses are destroyed by apoptosis.

3. Cell destruction due to negative signals:

There are several negative signals within the cells that promote apoptosis. These include accumulation of free radicals, exposure to UV rays, X-rays and chemotherapeutic drugs.

Mechanism of apoptosis:

The programmed cell death may occur due to three different mechanisms:

1. Apoptosis due to internal signals.

2. Apoptosis triggered by external signals e.g. tumor necrosis factor-α (TNF-α), lymphotoxin.

3. Apoptosis triggered by reactive oxygen species.

Role of caspases in apoptosis:

A group of enzymes namely activated proteases play a crucial role in the programmed cell death. These proteases are actually cysteinyl aspartate specific proteinases or in short, commonly referred to as caspases. There are about ten different types of caspases acting on different substrates ultimately leading to cell death. For instance, capsase I cleaves interleukin 1β.

Inhibition of caspase activities:

Since the caspases are closely involved in apoptosis, it is possible to prevent cell death by inhibiting their activities. Certain specific peptides that can inhibit caspases, and thus apoptosis have been identified.

Measurement of Apoptosis:

A simple and easy way of detecting dead or dying cells is the direct microscopic observation. The dying cells are rounded with dense bodies which can be identified under phase contrast microscope. The cells that have undergone apoptosis contain fragmented chromatin which can be

detected by conventional staining techniques. In recent years, more sensitive and reliable techniques have been developed for measuring apoptosis.

Some of them are briefly described:

Determination ADP/ATP ratio:

Both the growth and apoptosis of cells require ATP. But when there is growth arrest, an elevation of ADP occurs. Thus measuring ADP/ATP ratio will throw light on the dead cells. In fact, some assay systems for measuring ADP/ATP ratios are commercially available.

TUNEL assay:

A significant biochemical event for the apoptosis is the activation of endogenous nuclease activity. This enzyme cleaves DNA into fragments with free 3-hydroxyl groups. The newly formed small DNA fragments can be extended by employing the enzyme DNA polymerase. If labeled nucleotides are used for DNA fragment extension, they can be detected.

TUNEL is an abbreviation for TdT-mediated dUTP nick end-labeling assay. TUNEL is very fast and effective for the determination of DNA fragments formed by endogenous nuclease activity. The apoptotic nuclei can be identified by a fluorescent technique using fluorescein isothiocyanate (FITC) and 4, 6-diaminophenylindole.

DNA laddering test:

During the course of apoptosis, the genomic DNA is cleaved to mono — and oligonucleosomal DNA fragments. These fragments can be separated by agarose electrophoresis, and detected. The nucleosomal fragments of apoptotic cells give a characteristic ladder pattern on electrophoresis.

Limitations of the test:

DNA laddering test is not very specific since several cells that have undergone apoptosis may not show DNA laddering. Further, some cells not subjected to apoptosis may also show DNA ladders, for these reasons, DNA laddering test is coupled with some other test for measurement of apoptosis.

As programmed cell death (PCD) or apoptosis has emerged as an important regulator of development and homeostasis in multicellular organisms, methods to quantify apoptosis and to distinguish it from necrosis have been developed. This unit presents a set of assays for these purposes, many of which are technically very simple and ideally suited to the study of hematopoietic cells. The first basic protocol allows the qualitative and quantitative assessment of apoptosis in lymphocyte cell cultures using light or fluorescent microscopy. Three protocols follow that are designed to detect nuclear DNA fragmentation and support protocols describe methods to radiolabel the DNA and cytoplasm of the cells to be tested. Techniques that quantitate apoptotic cells using flow cytometry are then described and support protocols provide methods for priming T cell clones and freshly isolated lymph node cells, respectively, for T cell receptor (TCR)-induced apoptosis. Quantitative detection of DNA fragmentation in apoptotic cells is also described. TdT-mediated dUTP-biotin nick end-labeling (TUNEL) methods are provided for the detection of apoptotic cells, along with procedures for the flow cytometric quantitation of apoptotic cells using TUNEL, and TUNEL, staining of tissue sections to identify apoptotic cells. Since much remains incompletely understood about the molecular pathways of programmed death, and it is probably best to perform more than one of the basic protocols to confirm an observation of apoptotic cell death.

CELL VIABILITY ASSAY

Calculation of cell viability and the total number of viable cells are widely used methods in cell suspension preparation, for cell treatment with toxins, drugs, cytokines and for estimation effects of apoptosis triggering molecules. This is also important step when dose-response effect is

evaluated per cell number. In addition, determination of viable cell number is a start point in cell separation protocols regardless the separation method.

The most common assays for estimation of cell viability are based on cell membrane integrity and among them dye exclusion assay with trypan blue is widely used in routine laboratory work. Blue stained cells are dead cells

and the percentage of viable cells is calculated as ratio of viable (unstained) and total number of enumerated cells (dead and viable cells). Cell counting is commonly done using hemocytometer and classic light microscope.

In vitroassays for cell death determination ELECTRON MICROSCOPY INVESTIGATION OF APOPTOSIS

Apart from classical cytology, electron microscopy (EM) is used for investigation of apoptosis and necrosis in cultured cells. Electron microscopy gives excellent intracellular and ultrastructural cell characteristics on which one can study every stage of apoptosis. Electron microscopy studies simultaneously with classic cytology/histology provide the background for the formulation of the apoptotic cell death concept, which was proposed by Wyllie. This concept can be used until today. An apoptotic cell typically undergoes shrinkage (i.e., apoptotic volume is decreased), chromatin condensation, karyorrhexis, and the eventual budding of the plasma membrane into apoptotic bodies. These morphological changes are considered the gold standard for distinguishing this type of cell death. Conversely, oncosis is a passive catastrophic cellular event where marked swelling, aggregate organelle disruption, nd plasma membrane blebbing prevail. There is little or no evidence of chromatin remodeling during oncosis, and the cell rapidly succumbs to cytolysis. This cytolysis is end-stage cellular decay that is the defining feature of necrosis. Apoptotic cells will eventually lose plasma membrane integrity and become necrotic in vitro . However, this is not believed to occur with high frequency in vivo because apoptotic cells display signals (e.g., the externalization of phosphatidylserine (PS) on their plasma membrane) that encourage their expeditious removal by phagocytosis. However, EM

studies are not recommended for routine work due to expensive equipment and highly trained and experienced personal. Besides, high cell number is required for data interpretation especially when we exactly need to express degree of apoptosis, for example as apoptotic index (number of apoptotic cells per 1000 enumerated cells.

ASSAY FOR ESTIMATION OF DNA FRAGMENTATION

One of the hallmarks of apoptosis is DNA fragmentation. Commercial apoptosis detection kits (TUNEL assay) enable to study apoptotic cells in situ by specific end labeling of DNA fragments. Nucleotides labeled with either digoxigenin or fluorescein are enzymatically added to 3' hydroxyl DNA ends by deoxynucleotidyl transferase (TdT). This enzyme is more sensitive for apoptotic DNA fragmentation than to necrosis, and it is more specific that DNA polymerase. The antidigoxigenin antibody fragment carries either a conjugated reporter enzyme (peroxidase) or fluorescent molecules to the reaction site. The localized peroxidase enzyme then catalytically generates and intense signals from chromogenic substrate that can be observed using light microscopy, while fluorescein can be

observed by florescence microscopy or by flow cytometry. Large number of DNA fragments that appeared in apoptotic cells result in multitude of 3' hydroxyl termini. This is used to identify apoptotic cells by labeling the 3' hydroxyl ends with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). A substantial number of these sites are available in apoptotic cells providing the basis for the method utilized in the APO-BRDU kits. Data indicated that Br-dUTP is more readily incorporated into the genome of apoptotic cells than fluorescein, biotin or digoxigenin. Non-apoptotic cells do not incorporate significant amount of Br-dUTP. Comet Assay IV TM developed from Perceptive instrument, Laboratory Company, is widely recommended as an interactive live video-based system for scoring cells subjected to the single cell gel electrophoresis technique (comet assay). Comet Assay IV incorporates all major measurement parameters that are necessary for estimation of degree of apoptosis. Once the target number of cells or a particular slide have been scored, data can be saved to Microsoft Excel for

the next analyses. Using a high-definition video camera attached to the microscope in the system, Comet Assay IV can transfer a live video picture to computer monitor.

COLORIMETRIC ASSAYS FOR STUDY CELL DEATH

The cytotoxic assays described below are the most useful option for cell death investigation for everyday laboratory work, since they are inexpensive, easy for manipulation and obtained data are reproducible and comparable. Among these colorimetric assays, authors prefer assay based on determination of released intracellular molecule, lactate dehydrogenase (LDH). LDH is released through the altered cell membrane following cell death process. The assay principle is based on consideration that tumor cells possess high concentration of intracellular LDH. In the presence

of the drugs or cytokines that trigger cell death receptors superfamily tumor cells undergo apoptosis or necrosis. After cell membrane damage, LDH can be released and thus we detect death cells. For exact calculation of percentage of dead cells, it is needed to calculate the intracellular LDH amount in respect to the released LDH amount. LDH release assay is rapid and very sensitive. Significant LDH release from cultured cells depends on cell type (tumor or normal), cell number or cell separation process. Cultures of peripheral blood lymphocytes, separated from healthy volunteers or many tumor cells (K562, Raji, HeLa, PC-MDS) after in vitro treatments with TNF, showed significant dose-dependent increase in LDH activity. In addition, LDH is mostly released in comparison

to other intracellular enzymes, and it is useful since it represents anaerobic type of tumor cell metabolism.

The determination of spontaneous LDH release in cell supernatants is a very appropriate for the estimation of natural killer (NK) cell death in evaluation of innate immunity, safety of vaccine application in vitro and in vivo, and for virus toxicity on cultured cells. Using LDH release assay we can detect minimal membrane damage. This assay is also widely recommended as non-radioactive, rapid (2 h versus 4 h) and safety one in replacement of classical

radioactive chromium release assay for estimation of NK cell activity, Sulforodamine B assay (SRB) is other colorimetric assay commonly used for estimation of cell sensitivity to cytotoxic agents. It is based on determination of total protein content in cultured cells before and after drug application.

SRB assay can serve to determine the percentage of cell growth inhibition in cultured cells as well as cell percentage of cytotoxicity.

CASPASE ACTIVITY ASSAYS

All typical signs of apoptosis are the result of activity of a complex biochemical cascade of events that execute cell proteolysis. Apoptotic signaling mainly converges in the activation of intracellular caspases, a family of cysteine-dependent aspartate-directed proteases, which propagate death signaling by cleaving key cellular proteins. Currently, 14 members of the caspase family have been identified, and 7 of them mediate apoptosis. Several assays were developed to study these molecules. One assay for caspase detection is based on spectrophotemetric measured such as previously described, but determined chromophore p nitroanilide (pNA) after cleavage from the labeled substrate YVAD-pNA. The pNA light emission can be quantified using a spectrophotometer or micro plate reader.

Comparison of the change in absorbance from apoptotic samples with controls allows determination of the fold increase in caspase activity as easy option. This assay is semi quantitative but for quantification of caspase activity, we have also flow cytometry or western blot techniques.

ANNEXIN V/PROPIDIUM IODIDE (ANNV/PI) ASSAY

This assay is based on the estimation of cell membrane changes during apoptosis and ability of the protein annexin V to bind to phosphatidylserine exposed on the outer membrane leaflet in apoptotic cells. In viable cells,

phospahtidilserine is located in the inner membrane leaflet, but upon induction of apoptosis, it is translocated to the outer membrane leaflet and becomes available for annexin V binding. However, phosphatidylserine is also appears

on the necrotic cell surface. Using of simultaneous combination of annexin V and propidium iodine (PI) there are different option to discriminate apoptotic from necrotic cells. The addition of PI enables that viable (AnnVneg/PIneg), early apoptotic (AnnVpoz/PIneg), late apoptotic (AnnVpoz/PIpoz) and necrotic (AnnVneg/ PI poz) cells can be distinguished. This assay requests flow cytometry for results interpretation. Flow cytometry technique enable expression analysis of several cell surface molecules and have a great application in hematology. However using PI/annexin as s double stained system we can also determined viable cells. Data acquisition and analysis by flow cytometry use computer system for determination events on membrane or changes in nucleus on separated flow cells in suspension. The acquisition of events is performed using software. Analysis can be completed after a previously fixed total number of events acquired. Debris cells need to clearly discriminate from nonviable cells.

Medical Ethics and Safety Measures in Culture Techniques:

Since the culture techniques involve the use of animal or human tissues, it is absolutely necessary to follow several safety measures and medical ethics. In fact, in some countries there are established legislation/norms for selection and use of tissues in cultures. For example, in United Kingdom, Animal Experiments (Scientific Procedures) Act of 1986 is followed.

The handling of human tissues poses several problems that are not usually encountered with animal tissues. While dealing with fetal materials and human biopsies, the consent of the patient and/his or her relatives, besides the consent of local ethical committee is required. Further, taking any tissue (even in minute quantities) from human donors requires the full consent of the donor in a prescribed format.

The following issues need to be fully considered while dealing with human tissues:

1. The consent of the patient and/or relatives for using tissues for research purposes.
2. Ownership of the cell lines developed and their derivatives.
3. Consent for genetic modification of the cell lines.
4. Patent rights for any commercial use of cell lines.

In the general practice of culture techniques using human tissues, the donor and/or relatives are asked to sign a disclaimer statement (in a prescribed proforma) before the tissue is taken. By this approach, the legal complications are minimized.

Safety measures:

Handling of human tissues is associated with a heavy risk of exposure for various infections. Therefore, it is absolutely necessary that the human materials are handled in a biohazard cabinet. The tissues should be screened for various infections such as hepatitis, tuberculosis, HIV, before their use. Further, the media and apparatus, after their use must be autoclaved or disinfected, so that the spread of infections is drastically reduced. Special legal and ethical problems

Legal aspects

In countries with established transplant programs, organ transplantation is highly regulated. Of particular concern is organ donation, with legal, medical, and social issues surrounding the procurement of organs, without compensation, for transplantation. Many of those issues are overcome by organ registries, in which individuals choose to become organ donors. Through such registries, donors can indicate which organs they are willing to donate upon death. Whether a person is a registered organ donor can then be indicated on a personal identification card (e.g., a driver's license), authorizing organ procurement once the individual is deceased. In the absence of legal consent via registration as an organ donor, organ procurement representatives are required to consult with next of kin for authorization to obtain organs from the deceased person.

Ethical considerations: Defining death

Transplantation raises important ethical considerations concerning the diagnosis of death of potential donors, and, particularly, how far resuscitation should be continued. Every effort must be made to restore the heartbeat to someone who has experienced sudden cardiac arrest or to restore breathing to someone who cannot breathe. Artificial respiration and massage of the heart,

the standard methods of resuscitation, are continued until it is clear that the brain is dead. Most physicians consider that beyond this point efforts at resuscitation are useless.

In many countries, the question of how to diagnose brain death—that is, irreversible destruction of the brain—has been debated by neurologists and other medical specialists. Most of these experts agree that when the brainstem is destroyed, there can be no recovery. The brainstem controls the vital function of breathing and the reflexes of the eyes and ears, and it transmits all information between the brain and the rest of the body. Most countries have established strict guidelines for how brainstem death is to be diagnosed and what cases are to be excluded—for example, patients who have been poisoned, have been given drugs, or have developed hypothermia. The neurological signs of brainstem death must be elicited by a trained clinician who is not concerned directly with the transplant operation. These signs are reverified after an interval, and, if there is the slightest doubt, further reverifications are made until the criteria are unequivocally met. The guidelines are not seriously disputed, and there has never been a recovery in a case that fulfilled the criteria of brainstem death.

Shortage of donors

Another area of ethical concern is the dilemma posed by the shortage of donor organs. Advances in immunosuppressive therapy have put increasing pressure on the supply of donor organs, and medical personnel sometimes find themselves having to determine who among the potential recipients should receive a lifesaving graft. Furthermore, there is a danger of commercial interests becoming involved with people willing to sell their organs for personal gain, and there is definite risk of illegal organ trafficking, in which organs are procured from unwilling donors and then sold to facilities that offer transplant services.

Possible Questions

2 Marks

1. What is Cell and Tissue Culture?
2. How Are Cell Cultures Obtained?
3. Write about primary culture
4. Explain finite and non-finite cell lines
5. Explain passage cell line
6. Write about the techniques involved in disaggregating the tissue
7. Explain enzymatic disaggregation of tissue
8. Explain the applications of cell culture
9. Write about organ culture
10. Explain subculture

8 Marks

1. Mention the types of disaggregation
2. Explain about cell synchronization
3. Explain 3D culture
4. What is cryopreservation. Explain it.
5. What are the biology and characterization of cultured cells.
6. What are the measuring parameters of growth and apoptosis?

UNIT-III

SYLLABUS

Molecular cell techniques: cell transformation- physical, chemical and biological methods; manipulation of genes; cell and organism cloning; green fluorescent protein and its application. Gene therapy.

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Cell transformation

Transformation broadly refers to the change in phenotype of a cell due to a new genetic material. As regards the cultured cells, transformation involves spontaneous or induced permanent phenotypic alterations as a result of heritable changes in DNA, and consequently gene expression. Cell transformation due to changes in the genetic material, and cell cloning involving the production of a population single cell.

Physical Methods of Gene Transfer | Genetics

The following points highlight the ten main physical methods of gene transfer. The methods are: 1. Biolystic or Particle Bombardment 2. Electroporation 3. Microinjection 4. Pollen Transformation 5. Liposome Mediated Transfer 6. Microlaser 7. Macro-Injection 8. Silicon Carbide Fiber (SCF) Mediated Transfer 9. Poly Ethylene Glycol (PEG) Mediated Transformation 10. Ultrasound Mediated Transfer.

Method # 1. Biolystic or Particle Bombardment:

One of the most spectacular successes in transformation of broad range of plants devoid of discrimination is the biolystic or gene gun method. This method, undoubtedly, is in driver seat among several other proposed methods.

This is the combination of biological and ballistic method. Klein (1987) has emphatically described effective and versatile particle gum method for delivering nucleic acids into intact plant cells and eventually result in transient expression of foreign gene.

In particle bombardment mediated process, DNA coated micro-projectile is used to transform plant tissue. After being accelerated, micro projectile is propelled to pierce cell wall and membrane and enter intact plant cells. The micro projectile is small to penetrate the plant cell with limited damage and successfully introduce DNA or RNA.

Biolystic process has been used to transform larger tissue and organs such as shoot tip, leaves, callus, cotyledon, zygotic and somatic embryos. This technique was first developed in 1987, intended to transform cereals. Infact, the first genetically modified (GM) crop like maize contains Bt-toxin gene was produced by this method.

Gene Gun Design:

Particle bombardment is based on the development of gas flow system such as powder driven (PDS-1000) or helium driven (PDS-1000/Hc). Efficiency of the system depends on selection of target material, particle to be used as micro projectile and acceleration.

Transformation efficiency depends on the amount of DNA dosage delivered into the cell, for example, low amount of nucleic acid delivery results in low transformation frequency and similarly high amount of DNA delivered into the cell leads to high copy number transformation efficiency.

In order to accomplish higher transformation rate at lower DNA concentration, the choice of chemical to coat particle have been modified, in which calcium chloride and polyamines are replaced by aminosiloxanes.

Nature and Preparation of Micro-carriers:

In the basic design of particle gun, coating of DNA onto small dense particles known as micro-projectiles is required. Several chemically inert metal particles such as gold, tungsten, palladium and platinum are employed. The size of the particles may vary between 1 and 1.6 pm in diameter.

The size of gold and tungsten particles is generally between 1 and 1.5 pm and 1.2 and 4 pm, respectively. Micro-metals are initially subjected to ethanol and sterile water washing process. Micro carrier suspension is then stored at 4°C for tungsten and – 20°C for gold particles.

Once preliminary treatment is done, micro-particles are mixed with plasmid DNA. Fixing of DNA onto the particles is carried out by either using ethanol or $CaCl_2$ precipitation method. After precipitation, the particles are washed, resuspended and either dried or stored on ice as an aqueous suspension.

Bombardment Process:

Type I—The Original Gun Powder Charge Method:

This was originally proposed by Klein (1987) to transform epidermal cells of Allium cepa (Onion). In this method, tungsten particles of 4 pm in diameter is coated with genomic RNA of tobacco and placed on the front surface of a cylindrical nylon projectile (macro projectile) of diameter 5 mm and 8 mm in length.

The whole projectile is prepared as a suspension in 1-2 pi of water. A gunpowder charge, detonated with a firing device is used to propel (accelerate) the nylon projectile down the apparatus. The tungsten particles move towards the steel plate, designed to stop the movement of nylon projectile.

The steel plate allows the micro projectile to pass through 1 mm aperture of stop plate. Tungsten projectile leaves particle gun with an initial velocity of 430 ms^{-1}. This high velocity can be

estimated by chronograph. The target cells/tissues are placed 15-20 cm from the end of the device. Many cells are bombardment simultaneously and about 90% of the cells typically contain bombardment micro-projectiles (Fig. 14.15).

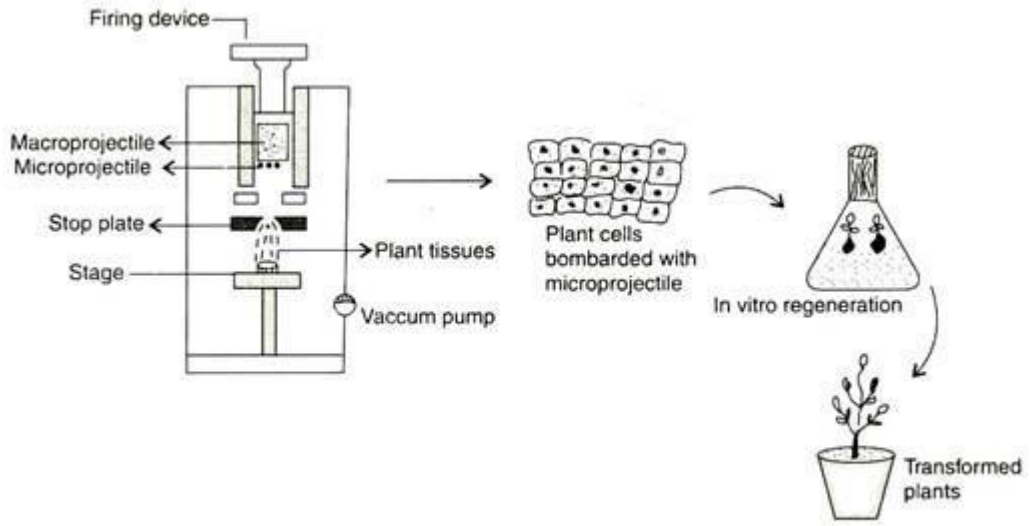


Fig. 14.15. Overview of microprojectile bombardment by gun powder device

Type II—Pressured Helium Gas Bombardment Device:

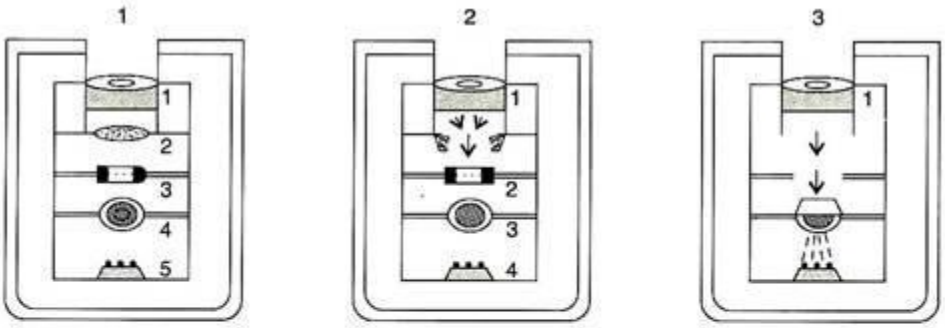
Helium blast device is a modified and upgraded version of tungsten gun powder discharge. This device was marked by BioRad as the 'PDS-1000/He' equipped with high-pressure helium as the source of particle propulsion.

The plasmid DNA-coated particle (micro-carrier) is placed on the front surface of the macro-carrier membrane and inserted into the apparatus. The plant tissue is placed into vaccum chamber, maintained at pressure 28 mmHg, just below the micro-carrier stopping plate. The stopping plate or macro-carrier retaining plate prevent the forward motion of the macro-projectile but designed to allow safe passage of only micro projectile.

Once partial vaccum is created at lower part of the ballistic device, pressure of the helium gas is accelerated to 1500 psi. Pressured helium gas is then released from the gas tank, and able to rupture the disc, which can resist the pressure upto 1200 psi. Following the burst of rupture disc, burst of helium gas is released. This propelled macro-carrier allows particles to move at high speed, and projectile down into a metal screen.

After macro-carrier impact with metal screen (stop plate), macro carrier is held back at stopping plate and allows micro-carrier to pass through lower chamber and finally hit plant material placed on the stage under partial vaccum. The shock wave generated after sudden release of pressured compressed gas and impact of macro projectile with stopping plate facilitate successful movement of micro-carrier and enters the plant tissue.

Establishing vaccum in the lower chamber can reduce resistance to movement of micro-projectile by the air (Fig. 14.16). To optimize velocity of micro-projectile several parameters like distance between the stopping plate and plant material can be varied. Following bombardment plant material is transferred to suitable culture media and eventually plants are regenerated.



1. Helium tank
2. Rupture disc
3. Macro and microprojectile
4. Stopping plate
5. Stage containing plant material

Fig. 14.16 Schematic representaiton of particle bombardment process (PDS-1000/Hc) (1) Acceleration of helium pressure upto 1500 psi ; (2) sudden release of pressurized helium gas from the tank and rupture of disc and (3) propultion of macroprojectile along with microprojectile towards stopping plate and projectile bombarded plate tissues

Merits of Biolystic Device:

i. It is efficient and easy to handle.

ii. It can transfer genes into many cells due to multiple sites.

iii. Technique can be widely used to transform different plate material types such as culture cells, pollen, meristem, embryos, and somatic embryo. Hence, in vitro regeneration is feasible.

iv. Only cells present in the line of micro-projectile movement are killed.

v. Utility of technique can be extended to a wide group of plants including dicots and monocots.

Demerits of Biolystic Device:

i. Integration of high copy number DNA sequence into the chromosome.

ii. Equipment costly.

iii. Cell/tissue damage due to bombardment by uncontrolled velocity of micro-projectiles.

Method # 2. Electroporation:

Electroporation is well suited for the transformation of plant cells and protoplast. Extensive work has been carried out regarding transformation of cereals using protoplast. Both linear and circular DNA can be transformed into the plant tissue. Intact plant cells of monocots have been transformed by electroporation. During electroporation, protoplast or intact plant cells are taken in electroporation chamber fitted with parallel steel electrodes.

The chamber is initially filled with buffer containing DNA of interest and high initial field strength of 1000-1500 volts with a short decay time in microseconds in applied. Pulse is applied by discharge of the capacitor across the cell. Alternatively, successful transformation is also carried out, by passing low voltage strength with larger decay of time.

Once protoplast is pulsed with low or high voltage DNA then migrated through pores into the plasma membrane induced by high voltage, eventually integrated into the genome. Most of the cereals, particularly rice and wheat have been successfully transformed by electroporation. Even other tissues such as callus and immature embryos are suggested.

Several methods have been suggested to increase transformation efficiency. Utility of osmotic buffer was well documented. Incubation of target material in high osmotic buffer before or after electroporation may increase efficiency of the technique. Addition of spermidine induces condensation of DNA, which results in enhanced efficiency of electroporation.

Advantages:

i. Efficient transformation.
ii. Large number of transformed cells can be obtained.
iii. Production of transformants with low transgene copy number.
iv. Electroporated cells exhibit same physiological status after transformation.
v. Least number of cells deaths.
vi. Electroporation of tissue can reduce in vitro regeneration problem.
vii. Low equipment cost.

viii. Does not require experties individual.

Disadvantages:

i. Requirement of protoplast for cumbersome in vitro regeneration of plants from protoplast.

ii. Difficulties associated with regeneration of plants from protoplast.

iii. Rise of obtaining genetic variation in protoplast mediated regenerated plants.

Method # 3. Microinjection:

Transformation of higher plant cells by intranuclear microinjection has been emerged as an attractive approach in recent days. Genetic transformation of animals and insects using microinjection of embryos has been well established.

In plant system, however, protoplast is selected as favourable choice for microinjection. This technique has advanced into diverse applications in key areas like cell biology, genetics and transgenic field. Recently, microinjection is widely employed in cereal transformation.

Microinjection is a precise way of delivering genetic material into the target cells. Several workers have demonstrated the feasibility of microinjecting substances into specific cells. In order to understand intercellular transport, fluorescent dyes were microinjected. The mode of virus infection was elucidated by microinjection of viral particles into intact plant cells.

Microinjection involves direct physical approach in depositing DNA into specific target cells. Generally, microinjection requires micro-capillaries and microscopic devices to deliver DNA into cells in such a way that the injected cells survive the treatment and is able to proliferate in the cultural conditions.

During microinjection, plant protoplast or partially synthesized cells are fixed to glass coverslips with the help of poly L. lysine. Further process requires holding pipette and micromanipulator or micro-injector. If any cell type is reluctant to attach to cover slips by binding agent, holding pipette can be an essential factor in microinjection.

These cell types are firmly retained on fixed place by blunt holding pipette. The exogenous DNA of 1 pm is taken in micro-injector and the cells or protoplasts are firmly immobilized by holding pipette by exerting suction pressure. Microinjection containing approximate dosage of DNA is then directly delivered inside the cells.

In microinjection, it is possible to microinject 200-350 protoplasts intra nuclearly and transformation frequency has been demonstrated with 20-60% success (Fig. 14.17). By means of reference marking on the coverslip, it is possible to locate microinjected cells/protoplast by recording with a video camera, which enables to work more freely from one microinjected cell to next one without interception.

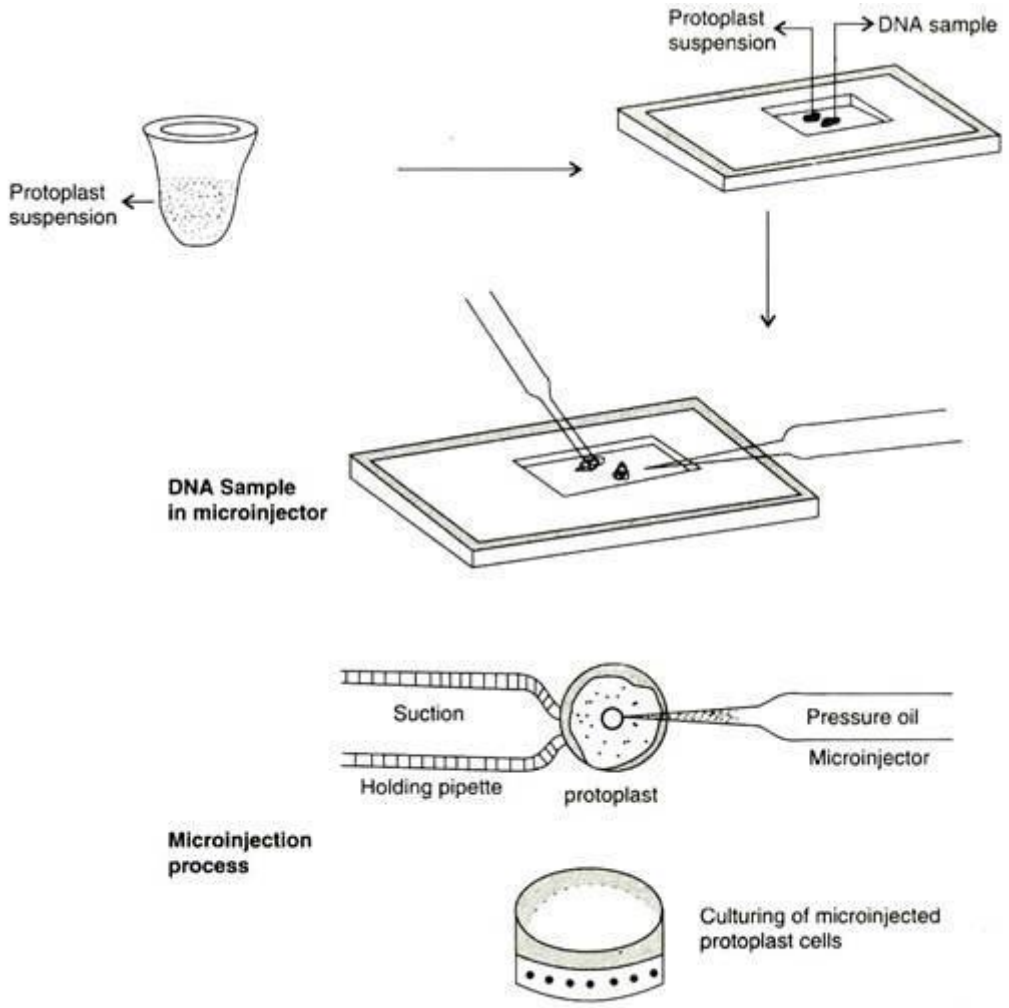


Fig. 14.17 Microinjection process

Earlier microinjection studies were restricted to insect fluorescent dye and introduction of virus. Microinjection of protoplast for transformation purpose is a recent achievement. This ensures delivery of 10^{-3} copies of plasmid DNA into the nucleus of a particular cell type. It was however, reasonably believed that injection of DNA directly into the nucleus accelerates transformation frequency.

Advantages:

i. The amount of DNA delivered can be optimized.

ii. Precise delivery of DNA. DNA delivery is predictable even into the cell nucleus.

iii. Small cell structures like microspores, callus and proembyros can be precisely targetted.

iv. Micro-culture is accomplished.

Disadvantages:

i. Only one cell receives DNA per injection.

ii. Handling of protoplast for microinjection requires skilled persons.

iii. Sophisticated equipment.

iv. Requirement of regeneration process from microinjected cells.

Method # 4. Pollen Transformation:

Pollen approach is ideal for gene transfer into plants. It is based on the prediction that DNA can be taken up into germinating pollen and can either integrate into the sperm nuclei to reach the zygote along with pollen tube. Several experiments in established laborataries with defined marker genes produced only negative results.

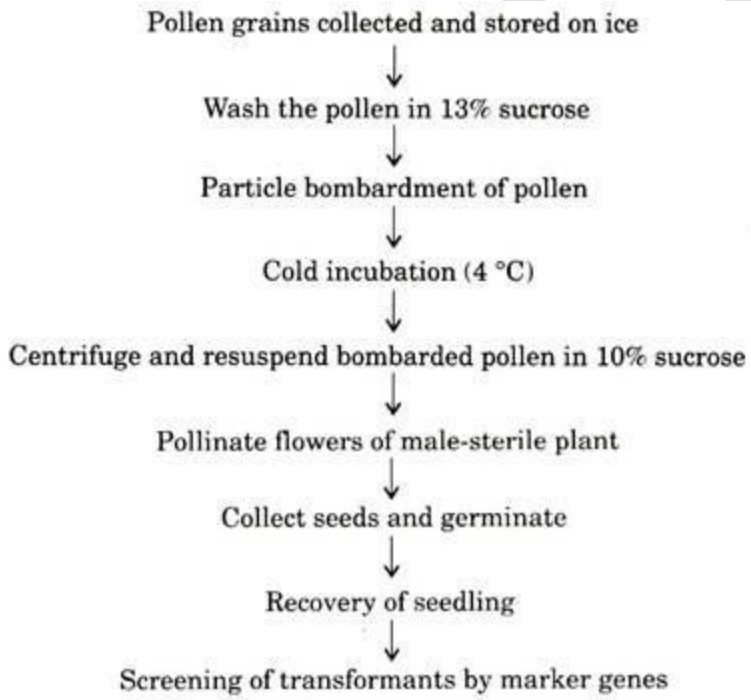
Subsequent experiments however, led to successful transformation with pollen grain. Direct delivery of DNA into pollen was used to obtain transgenic Alfa-Alfa i.e., Medicago sativa. In one of the classic experiments, plasmid bearing β-glucoronidase (GUS) reporter gene was introduced into the pollen by micro-projectile bombardment.

The bombarded pollen was found to express GUS activity, when flowers of male sterile plants are pollinated with bombarded pollen containing approximate gene produced fertile seeds. Thus, transforming pollen via particle gun would be advantageous since pollen is easily available and also free cells in large number.

Most of the pollen bombarded with small tungsten particles of size 1 to 1.2 μm at a target distance of 6 cm expressed high GUS activity. Bombarding with larger size tungesten particles (1.8 μm) decreases not only the number of pollen transformation but also their germination potential.

The process of DNA delivery into the zygote via pollen tube was found to be an effective approach. Selection of ovules for gene transfer is not feasible as it poses series of challenges in the isolation of egg. One of the main apprehension of pollen transfer in that the bombarded pollen may lose its germination potential because of the mechanical aberrations occurring on the membrane and cell organelle during penetration of tungsten with high velocity (28 inch of Hg).

Tobacco pollen was transformed with GUS via particle gun method. The transgenic tobacco expressed GUS activity efficiently and it was presumed that higher vaccum, chamber presents less air resistance to micro-projectile and cause deeper peneration into the cell and their oragenelle.



Method # 5. Liposome Mediated Transfer:

Liposomes are lipid vesicles, which are made artificially for transformation purposes. Liposomes are encircled by synthetic membrane of phospholipid. DNA-containing liposomes can be made

to fuse with protoplast and have also been applied to various tissues, cell cultures and even to pollen tube with the presumption that liposomes might aid in transporting DNA via plasmodesmata directly across cell walls.

When DNA containing liposomes are induced to fuse with protoplast using polyethylene glycol, get attached to protoplast membrane. Fusion of liposomes will be resulted at the point of attachment of DNA or plasmid DNA while entering the cell. This technique has no obvious advantages over any other gene transfer methods.

DNA containing liposomes can be directly microinjected into the vacuole, releasing the content of liposome into the cytoplasm. However, micro-injected vacuole led to fusion with tonoplast. This indicates that they could be used to transform even vacuolated cells. Although this method is elegant on certain criteria, unfortunately, regeneration plants are problematic with high vacuolated cells.

Method # 6. Microlaser:

Micro laser mediated gene transfer offers advantage only in specific cases where other methods are not advantageous. This technique involves focusing micro laser beam into the light path or microscope used to burn holes into the cell wall as membrane DNA uptake is possible through penetrated cells during incubation.

Several instances have shown that DNA gets adsorbed to the cell wall material even before its entry inside the cell.

Method # 7. Macro-Injection:

Gene transfer by macro-injection may not be an ideal choice on several occasions where size of injection needle exceeds cell diameter may disrupt it. DNA integration into cell would therefore require DNA to move into wound adjacent cells.

Entry of DNA may be impossible due to closer plasmodesmata and cell wall barrier. A marker gene, however, when injected into the stem below the floral meristem shows evidence of transformation. Due to lack of reproducible and convincing evidence, this approach was found to be highly limited.

Method # 8. Silicon Carbide Fiber (SCF) Mediated Transfer:

SCF does not require any specialized equipment. In this approach, silicon carbide fibres in average of 0.4-0.6 μm in diameter and 10-90 μm long are taken along with DNA in vortex tube. Plant cells or embryos are then introduced and vortexed gently. Entry of DNA into the cell is probably due to the penetration through the cell wall and plasma membrane.

Vortexing process results in the adhering DNA to silicon carbide fibres and gained access to inside the nucleus and eventually stable integration into the nucleus genome. Thus, passing of the DNA across the cell wall has advantage over other methods.

This approach does not involve regeneration of protoplast. Presently this technique is applicable to a particular species, which produce friable nature of callus. Many cereals cannot be transformed by SCF as they produce non friable brittle nature of callus.

Method # 9. Poly Ethylene Glycol (PEG) Mediated Transformation:

Poly ethylene glycol (PEG) is inert, least toxic to cells and protoplast. This was evidenced during somatic hybrid production. Efficiency of PEG has also been extended to gene transfer process. Protoplast can uptake naked DNA by treatment with poly ethylene glycol.

Efficiency of uptake can be increased in presence of divalent cation like calcium. PEG in complex with divalent cation can disturb molecular organization of the plasma membrane of the protoplast.

Positive charges of the calcium are attracted by the negative charge of the protoplast membrane and alter its zeta potential and destabilize it. Finally DNA makes entry inside the cell and integrates into the genome. The technique not only helps in assessment of transformation, but also involve in regulating gene transfer into the plant cells. Once DNA gains entry inside the cell, it is susceptible for degradation inside cytoplasm.

Method # 10. Ultrasound Mediated Transfer:

The uptake of foreign DNA by protoplast or cells can be facilitated by imposing ultrasound. Test tube containing cells or protoplast in a buffer is made to contact by inserting tip of ultrasonic device. The ultrasonic pulse generated by ultrasonicator of 0.4 m/cm^2 acoustic intensity is applied for 20-25 min.

Vigorous vibration in the medium and violent collpase of bubbles generates high hydrostatic pressure and shock wave may result in sporadic localized rupture in the membrane and it can facilitates uptake of exogenous DNA.

Introduction

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compoundsare introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell. An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

- Calcium phosphate
- DEAE dextran
- Cationic Lipid
- Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

Calcium phosphate mediated DNA transfer

Historical perspective

The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski (1962). They used total uncloned genomic DNA to transfect human cells deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Rare HPRT-positive cells with fragments of DNA containing the functional gene were identified by selection on HAT medium. Till then, the actual mechanism of DNA uptake was not understood. It was later found that successful DNA transfer takes place by the formation of a fine DNA/calcium phosphate co-precipitate, which first settles onto the cells and is then internalized. This technique was first applied by Graham and Van Der Eb in 1973 for the analysis of the infectivity of adenoviral DNA.

Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction. In this method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis

and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.

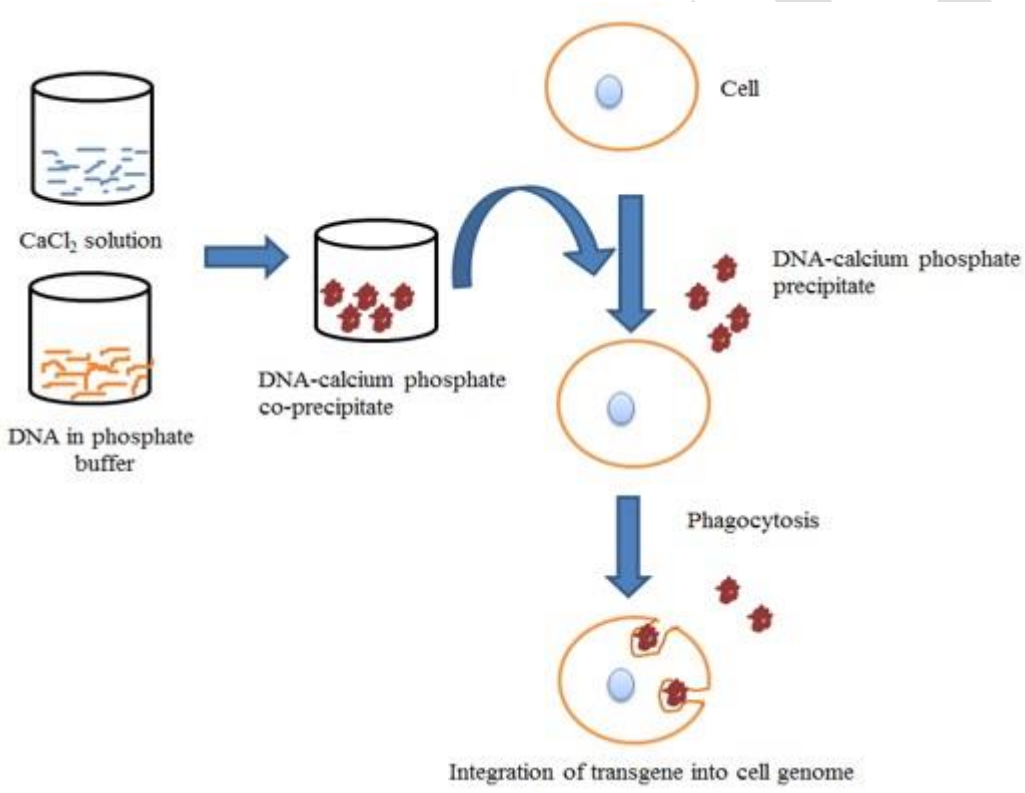


Figure: A schematic representation of transfection by Calcium Phosphate Precipitation.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.

- Can be used for stable ortransient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.

Optimal factors (amount of DNA in the precipitate, the length of time for precipitation reaction and exposure of cells to the precipitate) need to be determined for efficient transfection of the cells.

This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.
- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA

delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.

- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfection but not for stable transfection
- Typically produces less than 10% delivery in primary cells.

Another polycationic chemical, the detergent Polybrene, has been used for the transfection of Chinese hamster ovary (CHO) cells, which are not amenable to calcium phosphate transfection.

Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of 0.1 to 10 micrometer or 20-25 nanometers respectively.

- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

- Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

Negatively charged liposomes

- Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions

between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.

- However, formation of lipoplex, a complex between DNA and anionic lipids can occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which can neutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.
- They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro*gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

Liposome Action

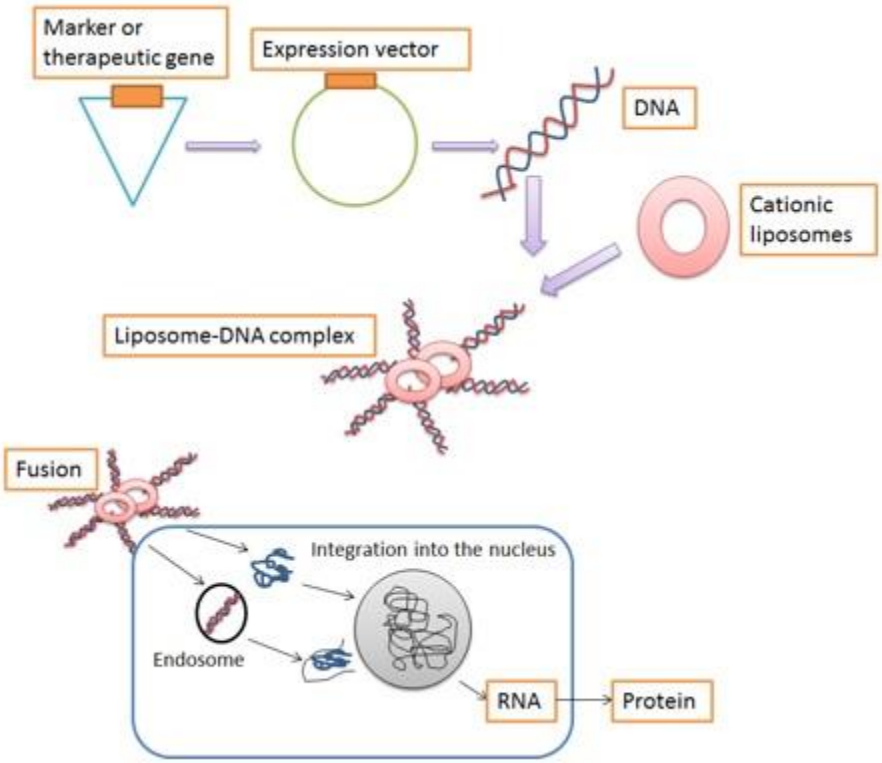


Figure5-2.1.3: Schematic representation of liposome action in gene transfer. *(Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)*

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to thespecific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell's lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.

- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

Other Methods

Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers.

5-2.1.4.1. Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.
- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.
- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.
- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bonds in the reducing environment of the cytoplasm.

Chitosan

- .

5-2.1.4.3. Polyphosphoester

- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications. They may result in extracellular persistent release of the DNA molecules thus enhancing the expression of transgene in the muscle as compared to naked DNA intake.
- Several polyphosphoesters with positive charges both in the backbone and in the side chain can be used as non-viral gene carriers.
- They can efficiently bind and protect DNA from nuclease degradation.
- They exhibit a significantly lower cytotoxicity than Poly-L-Lysine or polyethylenimine both *in vitro* and *in vivo.*
- It is a cell type dependent transfection method the efficiency of which can be enhanced using chloroquine.
- The transfection using polyphosphoesters is found to be effective in many cell lines, with some of them comparable to Liposome-mediated transfection.

Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.

- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.

- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.

- Once inside the cell, these complexes are then transported to the endosomes where these are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.

- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.

Introduction

The main gene transfer methods using biological means are as follows:

- Bacterial gene delivery i.e. bactofection.
- Delivery using a viral vector i.e. transduction

Bactofection

It is a method of direct gene transfer using bacteria into the target tissue, organ or organism. Various bacterial strains that can be used as vectors in gene therapy are listed in Table. The genes located on the plasmids of the transformed bacterial strains are delivered and expressed into the cells. The gene delivery may be intracellular or extracellular. It has a potential to express various plasmid-encoded heterologous proteins (antigens, toxins, hormones, enzymes etc.) in different cell types.

Strains that are invasive and having better cell to cell spread are more efficient.

Vector	Target gene	Disease	Model
L. monocytogenes	IL-12	*L. major*-infection	*Mus musculus*
L. monocytogenes	CFTR	Cystic fibrosis	CHO-K1 cells
S. typhimurium	VEGFR-2 (FLK-1)	Various carcinomas	*Mus musculus*
S. choleraesuis	Thrombospondin-1	Melanoma	*Mus musculus*
S. typhimurium	IFNϒ	Immunodeficiency	*Mus musculus*
S. typhimurium	CD40L	B-cell lymphoma	*Mus musculus*

Table 1 Bactofection in various disease models.

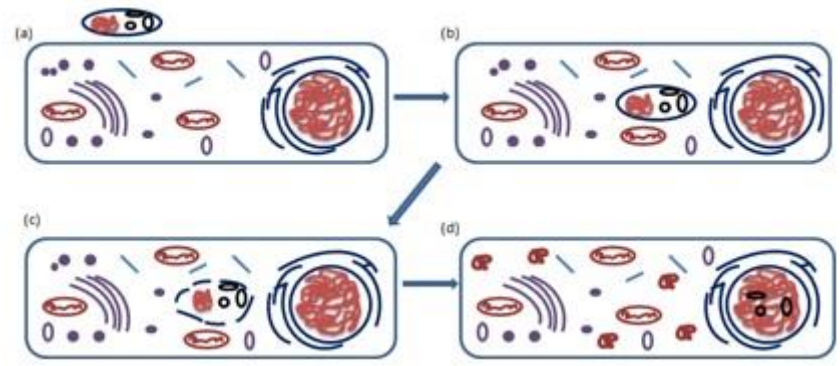


Figure 5-1.2: The process of bactofection (a) the transformed bacterial strain with plasmid containing transgene is transferred to target cell (b) genetically engineered bacteria penetrates into the cell (c) In the cytoplasm, the vector undergoes lysis and get destructed releasing plasmids (d) The released plasmids enter into the nucleus where the transgene is expressed by eukaryotic transcription and translation machinery

The efficiency of bactofection mediated gene transfer can be increased using integrin receptors. Integrin receptors are the transmembrane surface receptors present on the mammalian cell

surface. Another method, lipofectamine-mediated bactofection has also been employed for enhancing the gene transfer efficiency in *E. coli* strains, particularly in the transfer of large intact DNA for gene expression. This method is also effective on various widely used bacterial vectors such as *L. monocytogenes* and *S. typhimurium*.

Uses

- Bactofection can be used for DNA vaccination against various microbial agents such as viruses, fungi, protozoans and other bacteria.
- It can be used in the treatment of several tumours like melanoma, lung carcinoma and colon carcinoma in mice.

Advantages

- Simple, selective and efficient transfection.
- Low synthesis cost and can be administered easily.

Disadvantages

- Unwanted side effects associated with host-bacteria interaction. This can be reduced by using genetically modified bacteria which contain suicide genes that ease the bacterial destruction and thus reduces the risk of clinical infections.

Transduction

This method involves the introduction of genes into host cell's genome using viruses as carriers. The viruses are used in gene transfer due to following features-

- Efficiency of viruses to deliver their nucleic acid into cells
- High level of replication and gene expression.

- The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene (Figure 5-1.3).

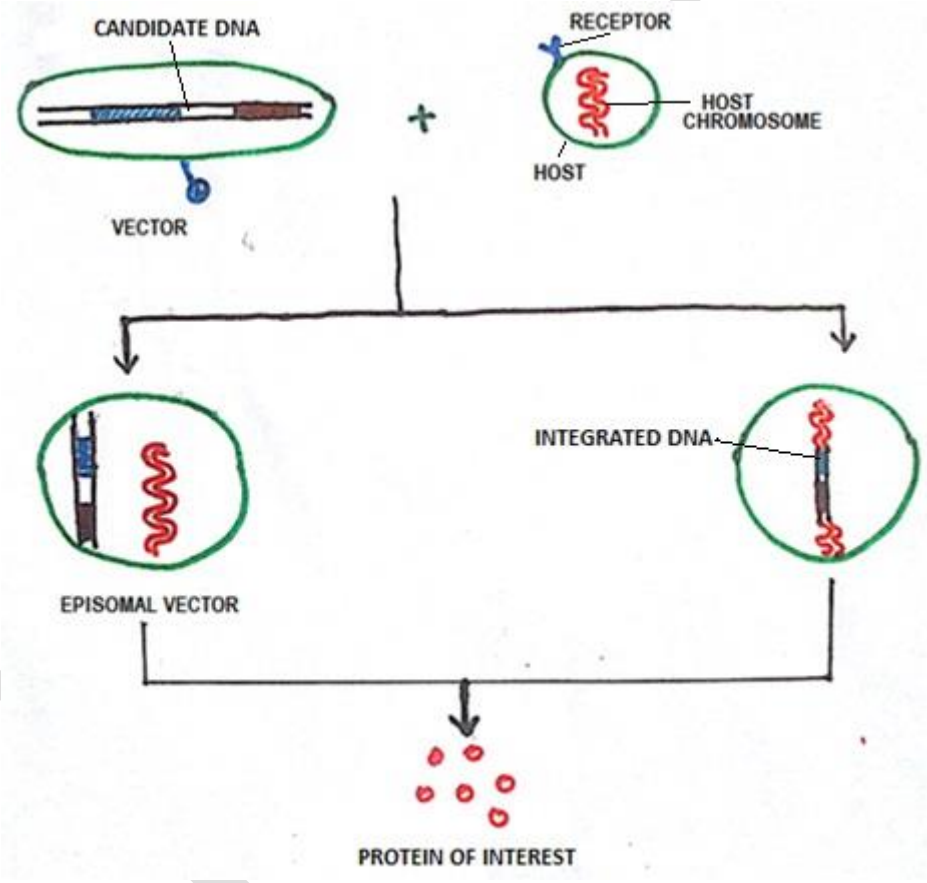


Figure 5-1.3: Transduction of a host cell.

Viral vectors as therapeutic agents

Viruses have paved a way into clinical field in order to treat cancer, inherited and infectious diseases. They can be used as vectors to deliver a therapeutic gene into the infected cells. They

can be genetically engineered to carry therapeutic gene without having the ability to replicate or cause disease.

Strategy for engineering a virus into a vector: The strategy for engineering a virus into a vector requires the following-

Helper virus

It contains all the viral genes essential for replication but lack the sequence coding for packaging domain (ψ) making it less probable to be packaged into a virion. It can be delivered as helper virus or can stably integrate into the host chromosomal DNA of packaging cell. Some vectors also possess the helper DNA lacking additional transfer functions to increase safety.

Vector DNA

It contains non-coding *cis* -acting viral elements, therapeutic gene sequences (up to 28–32 kb) and the normal packaging recognition signal allowing the selective packaging and release from cells. Some vectors comprise relatively inactivated viral genes as a wide type infection due to lack of other viral genes. The viral proteins essential for replication of the vector DNA are produced which then synthesize multiple copies of the vector genome (DNA or RNA, depending upon the type of vector). These structural proteins recognize the vector (psi plus) but not the helper (psi negative) nucleic acid resulting in the packaging of the vector genome into viral particles.

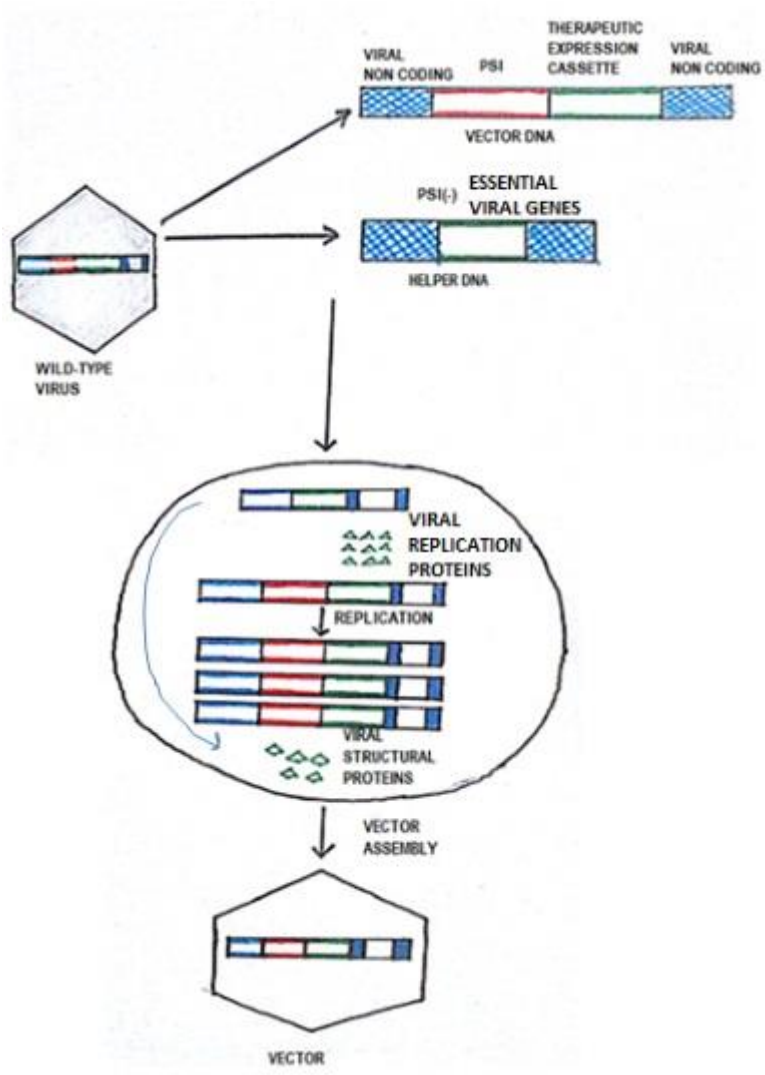


Figure 5-1.3.2(a): Strategy for engineering a virus into a vector.

Transgene may be incorporated into viral vectors either by addition to the whole genome or by replacing one or more viral genes which can be generally achieved by ligation or homologous recombination.

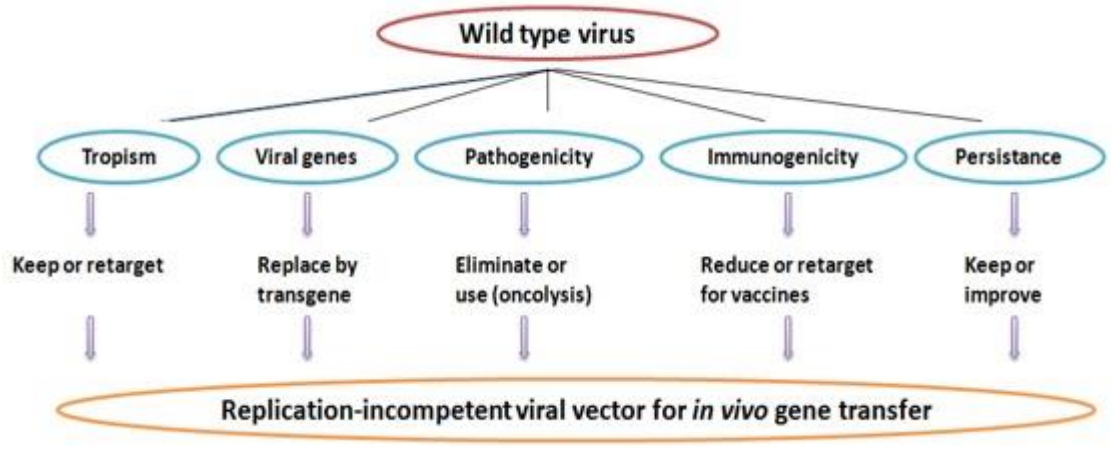


Figure 5-1.3.2(b): Modifications required for the generation of replication-defective viral vectors from wild type virus for *in vivo* gene transfer.

- If the transgene is added to the genome or replaces one or more non-essential genes for the infection cycle in the expression host, the vector is described as **replication-competent or helper-independent**, as it can propagate independently *e.g.* helper independent adenoviral vectors.

- However, if the transgene replaces an essential viral gene, this renders the vector **replication-defective or helper-dependent**, so that missing functions must be supplied *in trans.* This can be accomplished by co-introducing a helper virus or transfecting the cells with a helper plasmid, each of which carry the missing genes *e.g.* helper dependent retroviruses (Figure 5-1.3.2(b)).

- An alternative to the co-introduction of helpers is to use a complementary cell line, which is transformed with the appropriate genes called as ‘packaging lines'.

- The vectors from which all viral coding sequences have been deleted and depend on a helper virus which can provide viral gene products *in trans* for packaging and vector DNA replication are known as `*gutless vectors'* .

Advantages

- High capacity for foreign DNA
- The vector has no intrinsic cytotoxic effects.

Viral vectors

Various kinds of viruses can be used as viral vectors, but five classes of viral vector are used in human gene therapy-

1. Adenovirus
2. Adeno- associated virus (AAV)
3. Herpes virus
4. Retrovirus
5. Lentivirus

Vector	Host cells	Entry pathway	Vector genome forms	Transgene expression	Uses
Retrovirus	Actively dividing cells	Receptor-binding, membrane fusion	Integrated	Long term (years)	SCID, Hyperlipedemia, solid tumors
Lentivirus	Dividing and non-dividing cells	Receptor-binding, membrane fusion	Episomal	Stable	Hematopoetic cells, muscles, neuron, hepatocytes
Adeno	Most cells	CAR (Coxsackie and	Episomal	Transient	CNS,

virus		Adenovirus Receptor)-mediated endocytosis endosomal escape		(short term for weeks)	hepatocytes, pancreas
Adeno-associated virus	Most cells	Receptor-mediated endocytosis endosomal escape	Episomal (90%) Integrated (10%)	Medium to long term (year)	lung , muscle, heart, CNS
Herpes virus	Most cells	Endocytotic or membrane fusion	Episomal	Transient	Suitable particularly for nervous system

Table 5-1.4: Viral vectors and their properties.

Adenoviruses

- Adenoviruses are medium-sized (90-100 nm), non-enveloped, icosahedral viruses containing linear, double-stranded DNA of approximately 36 kb.
- 57 immunologically distinct types (7 subgenera) of adenoviruses cause human infections.
- They are unusually stable to physical or chemical agents and adverse pH conditions for long-term survival outside the body.
- There are six early-transcription units, most of which are essential for viral replication, and a major late transcript that encodes capsid.
- They result in transient expression in dividing cells as they do not integrate efficiently into the genome, but prolonged expression can be achieved in post-mitotic cells, like neurons.

- Adenoviruses are mostly attractive as gene therapy vectors, because the virions are taken up efficiently by cells *in vivo.* Adenovirus-derived vaccines have been used in humans with no reported side-effects.

The adenovirus infection cycle comprises two phases-early and late phase, separated by viral DNA replication. The first or "early" phase involves the entry of the virus into the host cell and virus genome to the nucleus. The late genes are transcribed from the major late promoter. The "late" phase involves the formation of gene products related to production and assembly of capsid proteins.

Construction of Adenoviral vectors

First generation adenoviral vectors were *replication deficient* , lacking the essential E1A and E1B genes and often the non-essential gene E3 and were called **'E1 replacement vectors'.** They had a maximum capacity of about 7 kb and were propagated in the cell lines transfected with DNA containing E1 genes e.g. human embryonic kidney line 293 (HEK 293).

Drawback

- These vectors caused **cytotoxic effect** due to low-level expression of the viral gene products, and chances of recombination between the vector and the integrated portion of the genome, resulting in the recovery of replication-competent viruses.

Higher-capacity vectors lacking the E2 or E4 regions in addition to E1 and E3 provide a maximum cloning capacity of about 10 kb but still allow low level of transgene expression. These must be propagated on complementary cell lines providing multiple functions. The use of E1/E4 deletions is a sound strategy as the E4 gene is responsible for many of the immunological effects of the virus.

To overcome the above limitations, an alternative strategy employs insertion of 'stuffer DNA' into the nonessential E3 gene as part of the vector backbone so to maintain

optimum vector size. Helper dependent adenoviral vectors (HDAd) are favoured for *in vivo* gene transfer due to deletion of all viral coding sequences.

Advantages of HDAd

- Large cloning capacity (up to 37 kb)
- High transduction efficiency
- Long term transgene expression
- Lack of immune response and cytotoxicity.

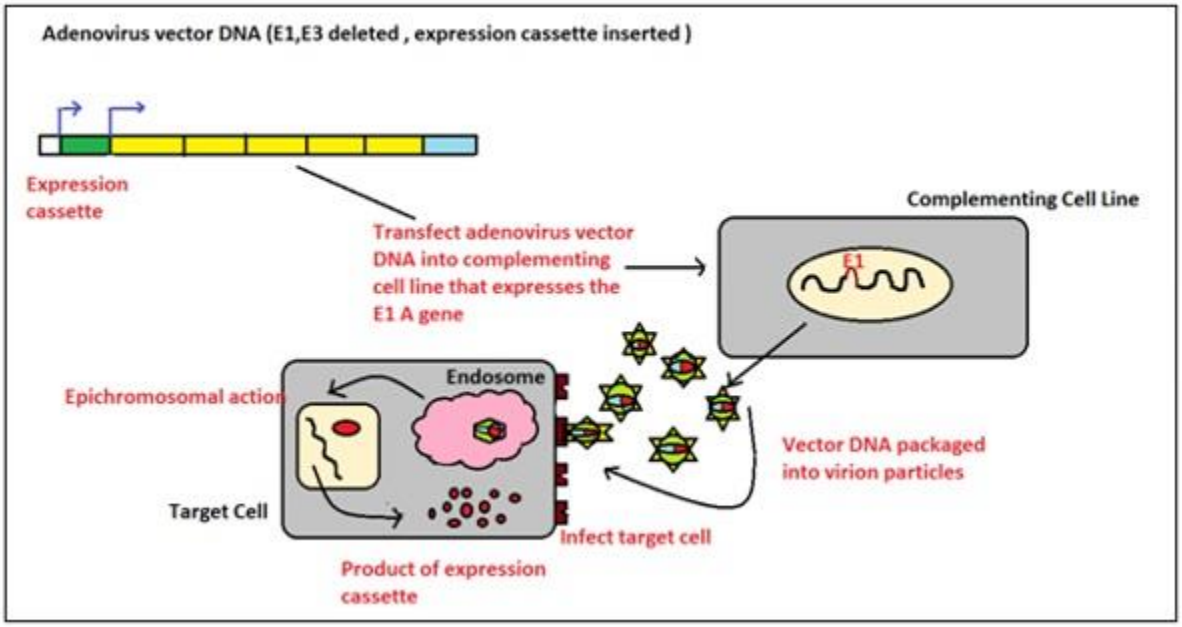


Figure 5-1.4.1(b):. Adenovirus vectors in gene transfer.

Role in gene therapy

- Mainly used for cancer treatment. Gendicine, a recombinant adenovirus, is the first gene therapy product to be licensed for cancer treatment.

Advantages of Adenoviral vectors

- High transduction efficiency
- Insert size up to 8 kilobases
- Generation of high virus titres
- High level of expression in a wide variety of cell types
- No mutagenic effects due to lack of random integration into the host genome.

Disadvantages of Adenoviral vectors

- Transient expression due to lack of integration into the host.
- Pathogenic to humans.

Adeno-associated virus

- It was first discovered as a contaminant in an adenoviral isolate in 1965.
- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family.
- It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection.
- In human cells, the provirus integrates predominantly into a 4-kb region (AAVS1) on chromosome 19. Subsequent infection by adenovirus or herpes virus can ‘rescue' the provirus and induce lytic infection.

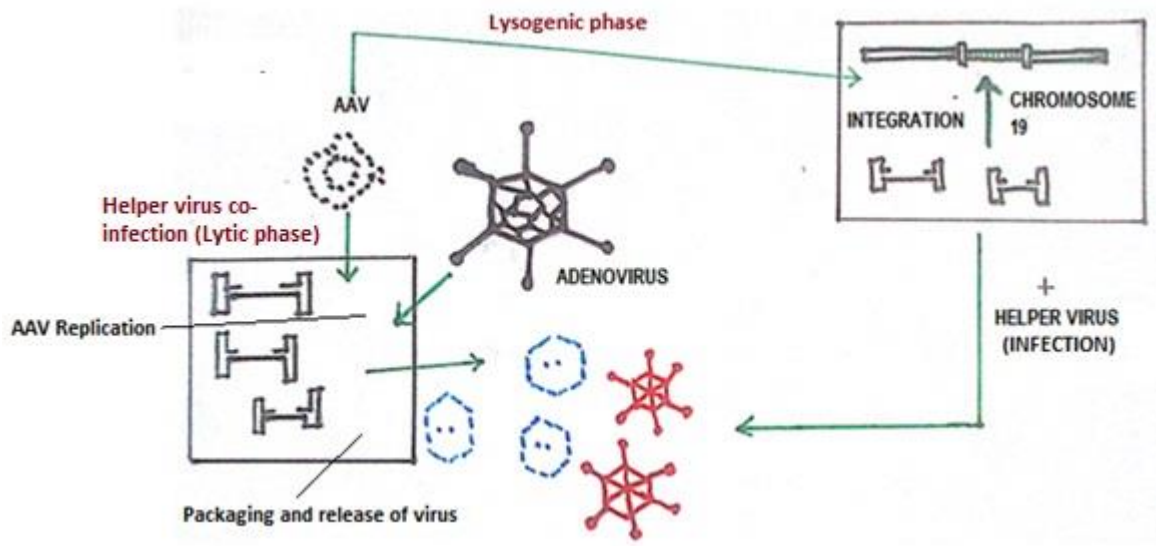


Figure 5-1.4.2(a): AAV life cycle.

AAV life cycle

- AAV life cycle comprises two phases-lytic and lysogeny.
- In the presence of helper virus, AAV undergoes lytic phase comprising genome replication, expression of viral genes and production of virions (Figure 5-1.4.2(a).).
- In the absence of helper virus, it undergoes lysogenic phase and integrates into the host cell's genome as a latent provirus. This latent genome undergoes replication by subsequent infection with helper virus.
- Both the stages of life cycle of AAV are controlled by complex interactions between the AAV genome andhelper virus, AAV and host proteins.
- **Adeno-associated viral genome**

  The AAV genome is small (about 5 kb) and comprises a central region containing rep (replicase) and cap(capsid) genes flanked by 145 base inverted terminal repeats (ITRs). The rep gene is involved in viral replication and integration whereas cap gene encodes

viral capsid proteins. ITRs are required for replication, transcription, proviral integration and rescue.

- In earlier AAV vectors, foreign DNA replaced the cap region and was expressed under the control of an endogenous AAV promoter. The transgene expression was inefficient using heterologous promoters due to inhibition of their activity by Rep protein.
- Rep interference with endogenous promoters resultedin cytotoxic effects of the virus. To overcome the above limitations, such vectors in which both genes were deleted and the transgene was expressed from either an endogenous or a heterologous promoter, were developed.
- In vitro manipulation of AAV is facilitated by cloning the inverted terminal repeats in a plasmid vector and inserting the transgene between them. Transfection of this construct into cells along with a helper plasmid produced recombinant viral particles.

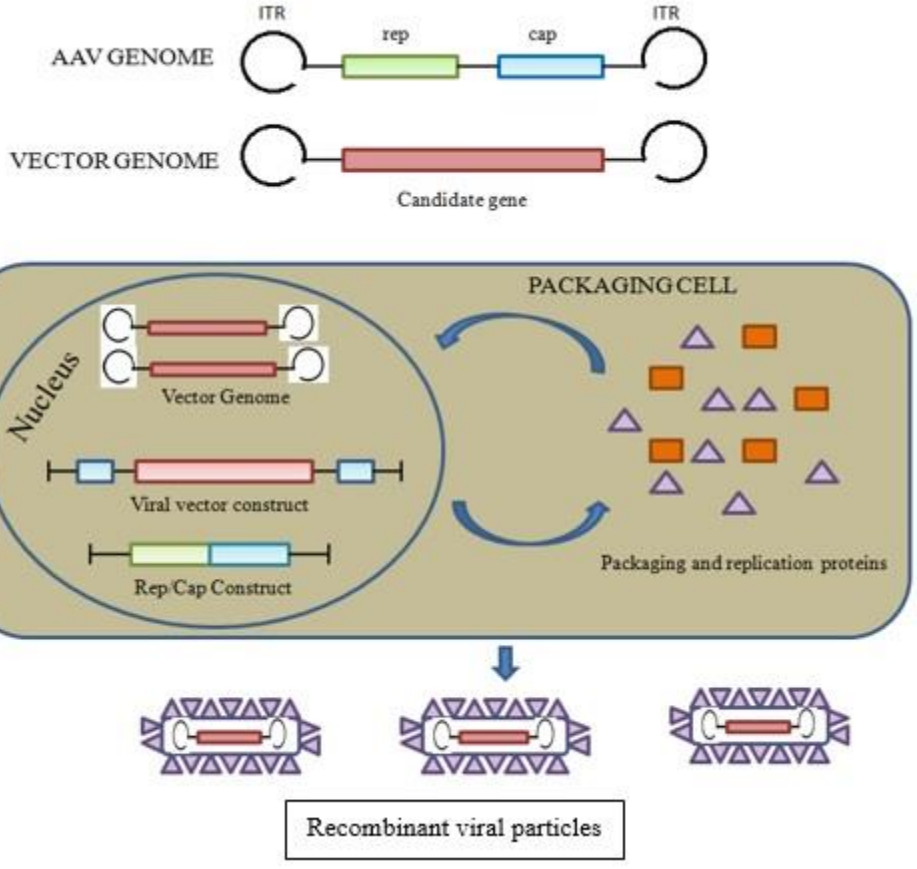


Figure 5-1.4.2(b): AAV Genome, Vector genome and Packaging coil.

Recombinant AAV (rAAV) is used as an expression cassette containing a reporter or candidate gene of interest. The foreign gene replaces all of the viral genes present in a wild type virus. Only the inverted terminal repeats are left to function as the essential replication/packaging signal.

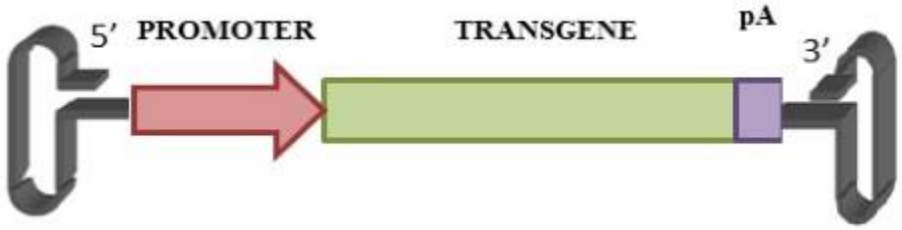


Figure 5-1.4.2(c): Organization of a typical recombinant AAV (rAAV) genome. pA represents Poly A tail.

Advantages

- Stable and have a wide host range
- Lack of initiating an immune response
- The dependence of AAV on a heterologous helper virus provides higher control over vector replication, making AAV vectors safer for use in gene therapy
- Potential of targeted/site-specific integration
- Non-pathogenic

Disadvantages

- AAV uses concatemeric replication intermediates
- They must be closely screened as they are often contaminated with adenovirus or Herpes Virus.
- Insert size is limited (4Kb)

- Difficult generation of high virus titres

Herpes virus vectors

• The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g.* EBV (Epstein–Barr virus) and the HSVs (Hepatitis B virus, e.g. HSV-I, varicella zoster).

• Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections.

• With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface.

• Unlike EBV as a replicon vector (contains both *cis* and *trans* acting genetic elements required for replication), HSV-I have been developed as a transduction vector for purpose of gene transferand can efficiently transduce a wide range of cell types.

• HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system. HSV can also be transmitted across neuronal synapses during lytic infections which can be used to trace axon pathways.

• Generation of recombinants in transfected cells takes place by homologous recombination. These viral vectors may be replication competent or helper dependent.

• The plasmid based amplicon vectors carrying only the *cis* -acting elements required for replication and packaging can be constructed. These vectors require packaging systems to provide the missing functions in *trans* .

Role in gene therapy

Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system).

Advantages

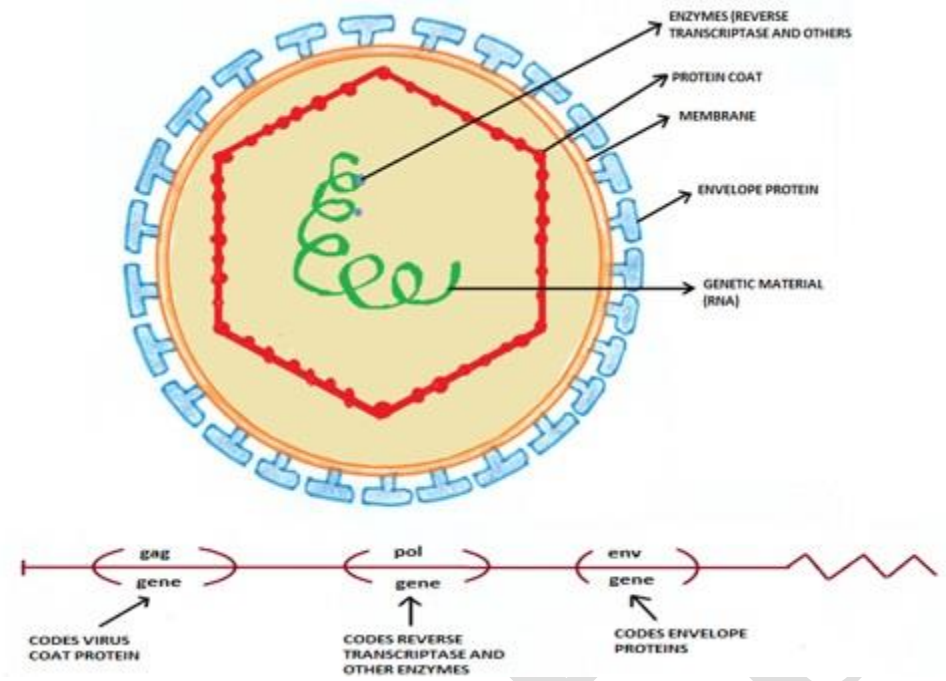
- Infects a wide range of cell types
- Insert size up to 50 kb due to large viral genome size
- Natural tropism to neuronal cells
- Stable viral particles allow generation of high virus titres (10^{12}pfu/ml)

Disadvantages

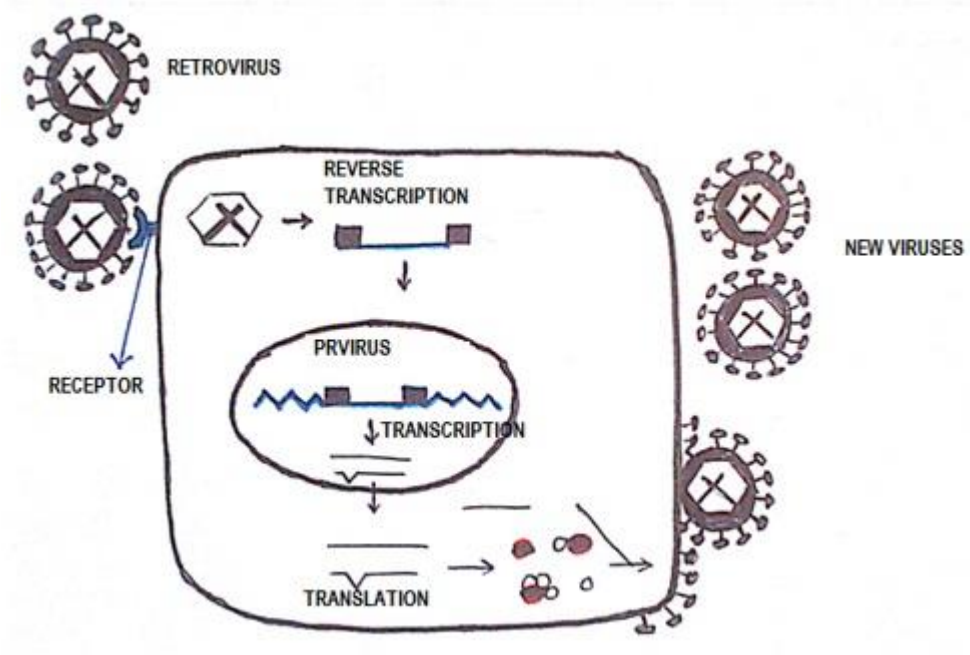
- No viral integration into host genome and transient transgene expression
- High level of pre-existing immunity
- Cytotoxicity effects
- Risk of recombination with latently HSV-infected cells

Retroviral vectors

Retroviruses are RNA viruses that replicate via a ds-DNA intermediate. The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell. The capsid contains two copies of the RNA genome, as well as reverse transcriptase/integrase. After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.

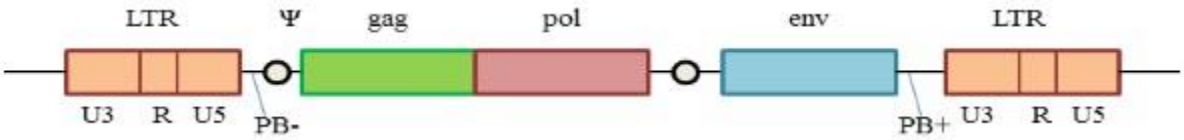


- **Figure 5-1.4.4(a):** Structure of a Retrovirus vector. RNA showed in the figure is single stranded.
- **Life cycle of retroviruses**
- A retrovirus, on binding to a cell surface receptor, enters the cell where it reverse transcribes the RNA into double-stranded DNA. Viral DNA gets integrated into the cell chromosome to form a provirus. Cellular machinery transcribes, processes the RNA and undergoes translation into viral proteins. The viral RNA and proteins are then assembled to form new viruses which are released from the cell by budding (Figure 5-1.4.4(b).).

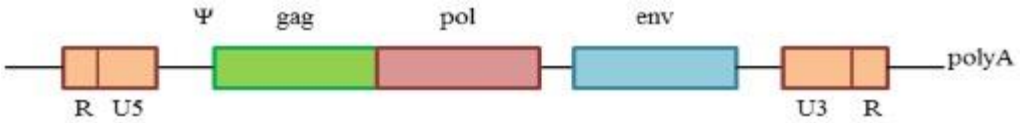


- **Figure 5-1.4.4(b):** Replication cycle of retroviruses.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511)*
- **Retroviral genome**
- The integrated provirus comprises three genes (*gag*, *pol* and *env*). The *gag* gene encodes a viral structural protein, *pol* encodes the reverse transcriptase and integrase and *env* gene encodes viral envelope proteins. Retrovirus can be classified as oncoviruses, lentiviruses, and spuma-viruses. Oncoviruses are simple whereas lentiviruses and spuma-viruses are complex retroviruses.
- Viral genomic RNA is synthesized by transcription from a single promoter located in the left LTR and ends at a poly-A site in the right LTR. Thus, the full-length genomic RNA is shorter than the integrated DNA copy and lacks the duplicated LTR structure. The genomic RNA is capped and polyadenylated, allowing the *gag* gene to be translated. The *pol* gene is also translated by read through, producing a Gag–Pol fusion protein, which is further processed into several distinct polypeptides. Some of the full-length RNA also undergoes splicing, eliminating the *gag* and *pol* genes and allowing the downstream *env*

gene to be translated. Two copies of the full-length RNA genome are incorporated into each capsid requiring a specific *cis* -acting packaging site termed ψ. The reverse transcriptase/ integrase are also packaged.

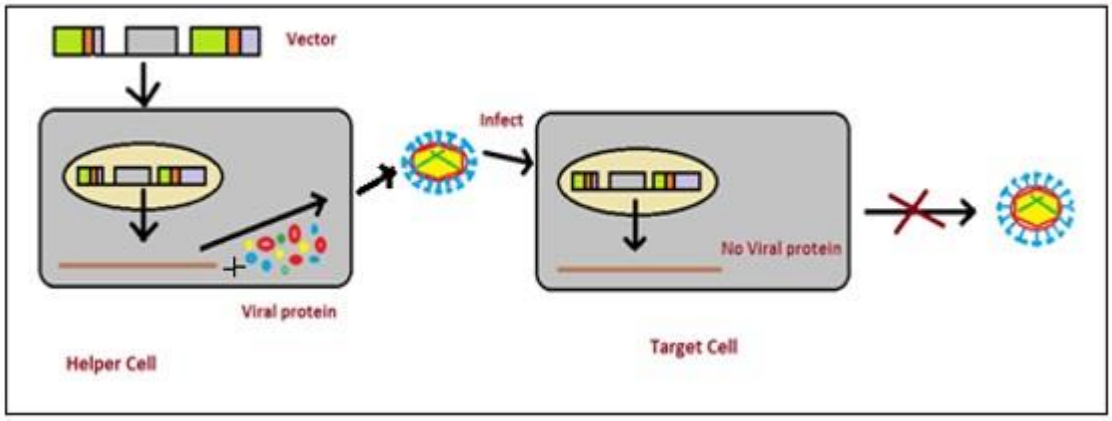


- **Figure 5-1.4.4(c):** An oncoretrovirus genome comprising long terminal repeats (LTRs) enclosing the three open reading frames *gag, pol* and *env*. PB represents primer binding sites in the viral replication cycle, ψ is the packaging signal and small circles represent splice sites.

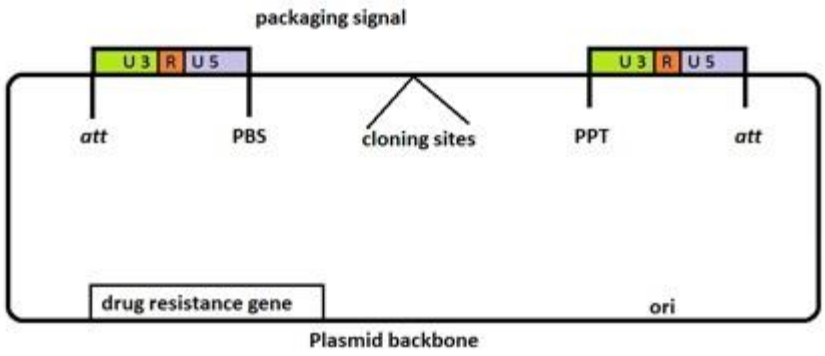


- **Figure 5-1.4.4(d):** Structure of a packaged RNA genome having a poly (A) tail but lacking the LTRs.
- **Construction of a retroviral vector and propagation in helper cell**
- The retroviral construct involved in gene delivery comprises two constructs-
- • A vector consisting of all *cis* -acting elements required for gene expression and replication (Figure 5-1.4.4(f).)
- • A helper cell expressing all the viral proteins (*gag*, *pol*, *env*) lacking in vector and support the replication of vector. Helper cell lacks RNA containing packaging signal which is required for formation and release of infectious particles but not for non-infectious viral particles.
- When the vector DNA is introduced into a helper cell, helper cell produces the viral proteins which help in the assembly of viral particles containing RNA transcribed from the viral vector. These viral particles on infecting the target cell, reverse transcribe the vector RNA into ds-DNA which gets integrated into the host genome forming a provirus which encodes the gene of interest. Target cells do not express viral proteins and cannot

generate infectious viral particles containing the vector RNA and thus cannot infect other target cells (Figure 5-1.4.4(e).).



- **Figure 5-1.4.4(e):** Propagation of retroviral vectors in helper cells.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511)*



- **Figure 5-1.4.4(f):** *cis* -acting elements required by a prototypical retroviral vector. The plasmid backbone contains a bacterial origin of replication (ori) and a drug resistance gene.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511.).*
- **Advantages**
- • Well studied system having high transduction efficiency
- • Insert size up to 8 kb

- • Integration into host genome resulting in sustained expression of the vector
- • Vector proteins are not expressed in host
- **Disadvantages**
- • Infection by retrovirus requires dividing cells
- • Low titres (10^6 -10^7)
- • Random integration
- • Poor *in vivo* delivery

MANIPULATING GENES

Gene transfer or genetic engineering involves the **transfer of** from **one species** of organism to **another species**, i.e.from a **donor** into a **recipient** organism. It is seen by some as a simple extension of other **biotechnological processes**, whereas to others it is considered as a development with much more sinister implications. In fact there has been pressure to use the term biotechnology, which has gained some public acceptance, to cover both.

A gene is a unit of hereditary information (i.e. it normally passes on characteristics from one generation to another), and is composed of .

Gene manipulation may be advantageous because it makes the resulting or organism easier to grow or manage, or to transfer a characteristic to a different crop, etc.

It **differs from selective breeding** which only involves members of the same species, in that usually only single genes are moved, often in addition to that organism's normal complement of genes (" "). Because selective breeding involves the normal methods of sexual reproduction (gamete transfer, fertilisation and development, etc.), it only results in large combinations of genes being transferred (the number of chromosomes contained in a gamete is in effect half a genome), and the effect of these genes may be masked or diluted due to by other genes.

Gene transfer techniques

In the laboratory, **specific enzymes** may be used to **cut** and**splice DNA**:

Restriction enzymes break DNA at specific parts of the molecule (nucleotide base sequences) - usually leaving so called "sticky ends". This can be done to both DNA from which genes are being taken, and to DNA in which genes are being inserted.

DNA ligase enzymes may be used to **rejoin such sections** into the other DNA.

The DNA containing the selected gene for the desired characteristic may then be inserted into cells of the target organism by means of **vectors** (here used in the service of Man, not disease organisms).

There are 2 main types of vectors: **plasmids** and **viruses**.

Gene transfer using plasmids

Agrobacterium tumefaciens is a bacterium which in nature causes plant disease - "**crown gall disease**" - but only in some dicots ("broad-leaved plants"), not in monocots (grasses and cereals).

A **gall** is a mass of undifferentiated plant tissue - similar to a cancerous tumour - produced in response to such an infection. Crown galls are usually produced on the stem just above the surface of the ground.
The bacterium contains a section of DNA called a **plasmid** in addition to its usual component of DNA. This Ti (tumour inducing) plasmid normally incorporates its DNA into the cells of the plant host ("integrating with their genome").

The ability of this organism can be utilised in **genetic engineering** to insert other genes into crop plants.

Sources of genetic material

It is thought that there is no technical reason why any characteristic in one species which is thought to be potentially useful in another species cannot be transferred by the application of these principles.

However, it is said that, due to commercial pressures, the main use of gene transfer to date has been to confer resistance to pests or diseases, rather than more direct impact on yield or other desirable characteristics. A gene thought to be useful may be obtained from a variety of sources, e.g.:-

The gene for **resistance to herbicide** may be obtained from (occasional) weeds which survive treatment with this chemical. This could perhaps usefully be incorporated into a crop which would then benefit from reduced competition from weeds, less hoeing etc, when sprayed with the appropriate herbicide. Commercially it would also mean that the seed and herbicide would be part of the same supply deal.
This procedure has actually been applied to crops of commercial significance, e.g. (beans), sugar beet, tobacco, and oilseed rape.

The *Bt* gene for production of **insecticidal toxin** from *Bacillus thuringensis* has been incorporated into several crops in order to protect them against insect pests.

Protection of crops from insect damage has also been tested using the gene for **venom** from **scorpions**!

Similarly, the effects of incorporating pest resistance genes from snowdrops into potatoes has been investigated.

Other novel ideas include the transfer of genes coding for important animal proteins such as the hormone insulin into plants, such as potatoes, which are easily grown and processed, and the transfer of genes into easily managed animals such as cows, sheep, etc, which may produce milk containing valuable proteins such as human antibodies and anti-cancer products.

Other alternative approaches involve isolation and modification of genes so that normal developmental changes do not occur. For example, there are several enzyme-controlled stages in the **ripening** and subsequent deterioration (**spoilage**) of **fruit**. Modification (inhibition) of the genes producing these enzymes can slow down the changes which occur after fruit is ripe. As a result, the **keeping quality** or shelf-life of the fruit is increased, and possibly the quality of products derived from these fruits is improved, as well as reducing the processing costs. This has been achieved and licensed in the case of **tomatoes** and products such as puree.

It is also said that attempts are being made to produce strains of soya beans which will flourish in temperate climates, and which are tall enough to facilitate mechanical harvesting.

Commercial implications of genetic modification of organisms

Interestingly, several **biotechnology companies** working in the field have attracted the attention of investors excited by the prospect of profits to be made. However, much **venture capital** has been used in the process, and there is considerable commercial rivalry and secrecy as to the exact details of the processes. Similarly, there is much public distrust as to the true intentions of workers in the field, and campaigners on each side have raised the profile of these activities in relation to regulatory authorities.

Recently there have been a variety of developments:

- Organisations representing consumer interests wish to ban GMOs (

) from entering the food supply chain, or to have it kept separate from other food supplies, and have its origin specifically stated in the product labelling

- chains have in some cases responded either by sourcing supplies of non genetically modified foods, or by identifying such ingredients in the labelling of the food, even if only a minor constituent. This is an ongoing development! Iceland was one of the first to do this, and on the 28th April 1999 Tesco also announced it was stopping using GMOs.

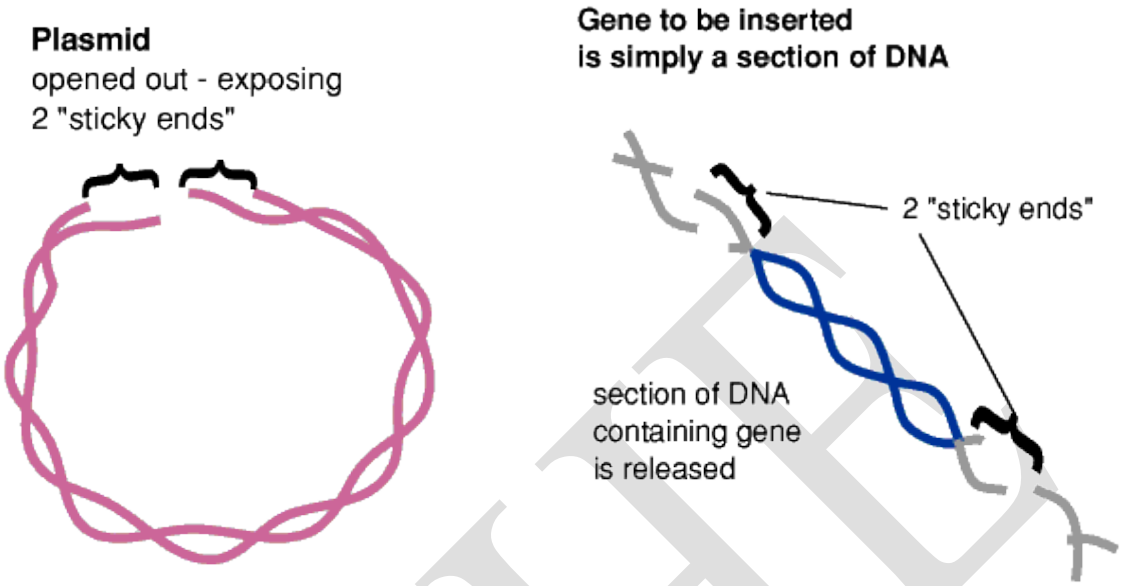
- or have mixed genetically modified foods with non genetically modified foods, either on the grounds that to do otherwise would increase costs, or in order to confuse the issue, in the hope of speedy acceptance of the product. This has been the case with **soya beans**, which are a major export from the USA.

- **Test plots** of varieties of plants being assessed for future use are covered by a variety of regulations designed to reduce the likelihood of any transfer of genes to surrou . In some cases corners have been cut and tempers have run high. Some pressure groups have advocated a moratorium on these trials, i.e. postponing them for several years.

- Farmers and growers must sign undertakings not to save seed from the crop for use to start another crop next year, because agrochemical companies have and other rights to the varieties used, and expect an exclusive agreement to use a combination of seed and control chemicals from the same supplier.
- The impartiality of some of the more important committees overseeing trials carried out by large companies has been called into question. Many of the decisions used to be made by employees of companies with interests in genetic modification, and a company owned by Lord Sainsbury, a government minister, holds patent rights to, and therefore profits from, important techniques in genetic manipulation.

Stages in the process of genetic modification - in more detail

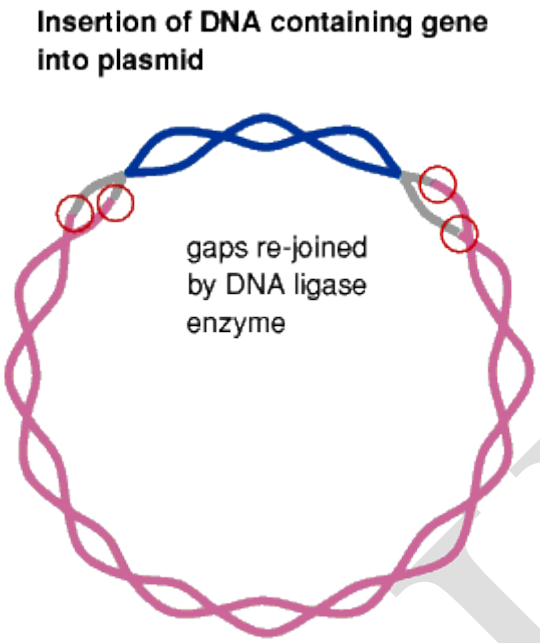
There are several methods of introducing the gene into the target organism.



DNA from the donor organism is broken up into short lengths with "sticky ends" using a .

One or some of these fragments should include the gene for the desirable characteristic, but often there is an element of chance, so the procedure is frequently repeated many times.

The tumour inducing which consists of DNA from *Agrobacterium tumefaciens* is similarly treated with the **same restriction enzyme**, opening out the circle of DNA leaving 2 ends. The presumed gene DNA is then mixed with the plasmid DNA, and conditions provided for the enzyme to work. In a number of cases, this will result in the plasmid re-joining, but with the gene incorporated into it.



The plasmid is reintroduced into the bacterium, which can then be grown up in large numbers by standard methods. When plants are infected with these bacteria, they will form galls of undifferentiated tissue, some cells of which will contain the required gene.

Sections of the gall may be encouraged to grow by special *plant tissue culture* techniques, possibly bulked up **in the lab** before conditions in the medium are changed to **encourage growth of roots and shoots**.

The resulting small plants may eventually be **potted up** and finally transferred to the **field**!

Other potential applications

The *Agrobacterium* situation has several parallels with **symbiotic nitrogen fixation**.

This also involves the activities of a species of **bacterium** (*Rhizobium leguminosarum)* which enters a **plant organ** (root) resulting in a **change in the plant cell** growth to form a **root nodule**, in which bacteria grow and perform chemical transformations.

It is hoped that genes for nitrogen fixation (*nif* cluster) may be transferred to non-leguminous plants. However there is more genetic information in these (12/20-30genes than can be easily transferred using plasmids, so more ambitious methods are being tried. Gene expression (turning them on) is a problem, especially as bacteria (prokaryotes - lacking nuclei/chromosomes) differ greatly from higher plants (eukaryotes - chromosomes protect DNA inside nuclei).

Viruses as vectors

Certain viruses can infect cells of animals and plants "without completing a destructive cycle" so they may also be used as gene vectors. They can usually carry larger portions of DNA than plasmids can .

An example is (lambda) phage - which can modify bacteria. DNA from a so-called **temperate phage** becomes incorporated into the DNA of its host: the bacterium *Escherichia coli (E. coli),* and can remain there indefinitely without having any harmful effect.

The phage DNA can be opened using restriction enzymes and foreign DNA may be inserted, so that the viral DNA can integrate with the host cells's "chromosome" (it is then called a *prophage*), and replicates with it at cell division.

Similarly, plant viruses may be used to transform plant cells genetically.

Other methods of gene transfer

These more drastic methods are mostly used with plant material, because the cell wall forms a barrier.

Ballistic techniques

Minute tungsten particles are coated with the DNA to be inserted, then shot into the target cells with an explosive charge. Apparently, this does not, however, cause significant structural damage to the cells.

Electroporation

In this technique, a brief pulse of **electric current** is passed through the cell, temporarily **increasing surface permeability** so that DNA is taken up from the surrounding liquid. This has been especially useful with pollen tubes and has resulted in the genetic transformation of seeds. Certain chemicals may have the same effect on the permeability of the cell wall.

Genetically modified organisms and food production

The same techniques used in the production of insulin and antibiotics may be applied to the use of genetically engineered bacteria in food production. Examples include **yeasts** with **high alcohol tolerance**, microbes with enhanced ability to digest waste straw, peat, coal, oil, etc., and improvements in capacity to produce valuable substances e.g. enzymes, flavourings, colourings. To some extent, industry has favoured the application of genetic modification processes to organisms which have achieved public acceptance, such as yeasts and **lactic acid bacteria** (*Lactobacilli*), which are responsible for cheese production as well as yoghurt and soy sauce.

Transgenic animals

There are obvious advantages in transferring genes for characteristics which are seen as desirable in the agricultural context, such as **resistance**to common animal **diseases**, lack of horns in cattle, and more **efficient growth** conversion, e.g. due to higher production of growth hormone, or greater digestive efficiency.

However, potential human **medical applications** have been seen to offer great opportunities. Production of blood clotting factor (needed by sufferers of the genetic condition) can be induced in the milk of sheep. So-called "designer milk" containig low cholesterol could probably find a profitable market.

More controversially, it has been said that transgenic organisms such as pigs could be used as sources of **organs for transplants** into humans, if human genes were transferred into these organisms at the embryo stage. This could reduce problems of rejection due to the immune

system of the donor. However, the risk of transfer of potentially very serious virus diseases from one species to another has become more obvious in the light of scrapie/BSE/CJD which is said to have "jumped the species barrier".

These possibilities pose **many ethical dilemmas**.

What is Cloning

Clones are organisms that are exact genetic copies. Every single bit of their DNA is identical.

Clones can happen naturally—identical twins are just one of many examples. Or they can be made in the lab. Below, find out how natural identical twins are similar to and different from clones made through modern cloning technologies.

How Is Cloning Done?

Many people first heard of cloning when Dolly the Sheep showed up on the scene in 1997. Artificial cloning technologies have been around for much longer than Dolly, though.

There are two ways to make an exact genetic copy of an organism in a lab: artificial embryo twinning and somatic cell nuclear transfer.

1. Artificial Embryo Twinning

Artificial embryo twinning is a relatively low-tech way to make clones. As the name suggests, this technique mimics the natural process that creates identical twins.

In nature, twins form very early in development when the embryo splits in two. Twinning happens in the first days after egg and sperm join, while the embryo is made of just a small number of unspecialized cells. Each half of the embryo continues dividing on its own, ultimately

developing into separate, complete individuals. Since they developed from the same fertilized egg, the resulting individuals are genetically identical.

Artificial embryo twinning uses the same approach, but it is carried out in a Petri dish instead of inside the mother. A very early embryo is separated into individual cells, which are allowed to divide and develop for a short time in the Petri dish. The embryos are then placed into a surrogate mother, where they finish developing. Again, since all the embryos came from the same fertilized egg, they are genetically identical.

2. 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.

How does SCNT differ from the natural way of making an embryo?

Natural fertilization, where egg and sperm join, and SCNT both make the same thing: a dividing ball of cells, called an embryo. So what exactly is the difference between the two?

An embryo's cells all have two complete sets of chromosomes. The difference between fertilization and SCNT lies in where those two sets come from.

In fertilization, the sperm and egg have one set of chromosomes each. When the sperm and egg join, they grow into an embryo with two sets—one from the father's sperm and one from the mother's egg.

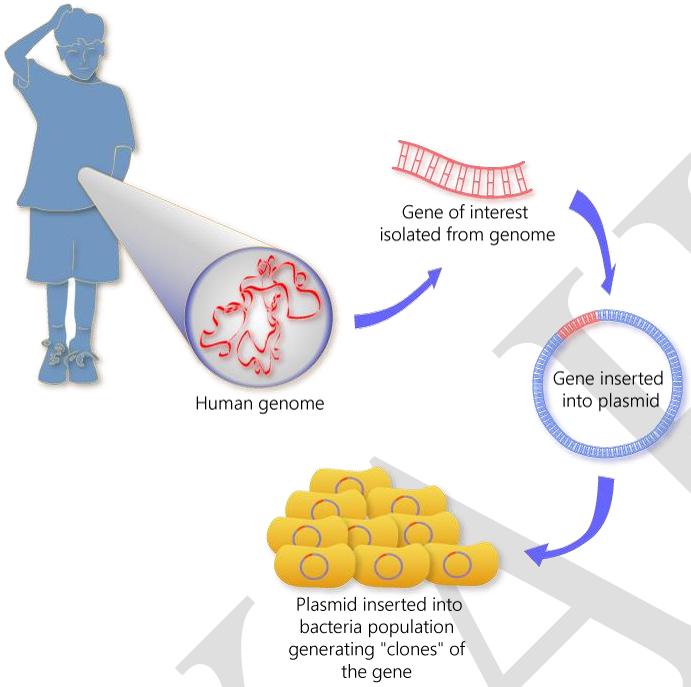
In SCNT, the egg cell's single set of chromosomes is removed. It is replaced by the nucleus from a somatic cell, which already contains two complete sets of chromosomes. So, in the resulting embryo, both sets of chromosomes come from the somatic cell.

Is cloning an organism the same as cloning a gene?

You may have heard about researchers cloning, or identifying, genes that are responsible for various medical conditions or traits. What's the difference?

When scientists clone an organism, they are making an exact genetic copy of the whole organism, as described above.

When scientists clone a gene, they isolate and make exact copies of just one of an organism's genes. Cloning a gene usually involves copying the DNA sequence of that gene into a smaller, more easily manipulated piece of DNA, such as a plasmid. This process makes it easier to study the function of the individual gene in the laboratory.



Organism Cloning

Organism cloning (also called **reproductive cloning**) is a method used to make a clone or an identical copy of an entire multicellular organism. Most multicellular organisms undergo reproduction by sexual means, which involves the contribution of DNA from two individuals (parents), making it impossible to generate an identical copy or a clone of either parent. Recent advances in biotechnology have made it possible to reproductively clone mammals in the laboratory.

Natural sexual reproduction involves the union, during fertilization, of a sperm and an egg. Each

of these gametes is haploid, meaning they contain one set of chromosomes in their nuclei. The resulting cell, or zygote, is then diploid and contains two sets of chromosomes. This cell divides mitotically to produce a multicellular organism. However, the union of just any two cells cannot produce a viable zygote; there are components in the cytoplasm of the egg cell that are essential for the early development of the embryo during its first few cell divisions. Without these provisions, there would be no subsequent development. Therefore, to produce a new individual, both a diploid genetic complement and an egg cytoplasm are required. The approach to producing an artificially cloned individual is to take the egg cell of one individual and to remove the haploid nucleus. Then a diploid nucleus from a body cell of a second individual, the donor, is put into the egg cell. The egg is then stimulated to divide so that development proceeds. This sounds simple, but in fact it takes many attempts before each of the steps is completed successfully.

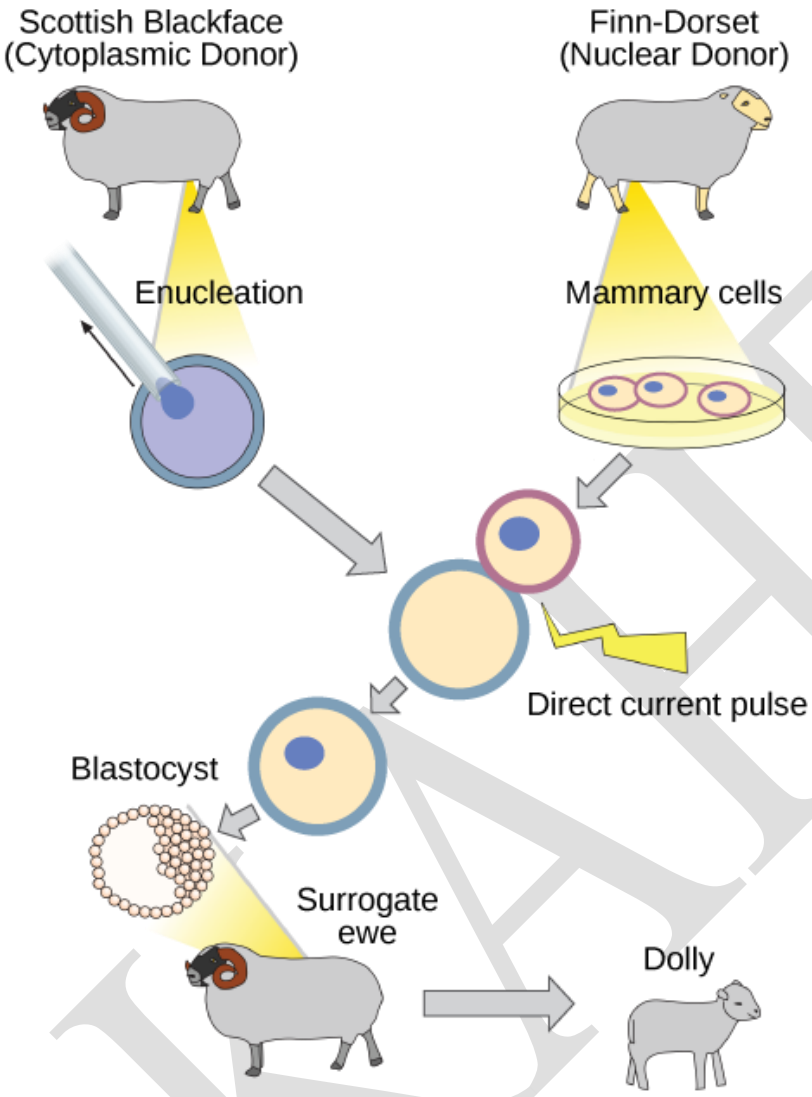


Figure 9-I-1. Dolly the sheep was the first agricultural animal to be cloned. To create Dolly, the nucleus was removed from a donor egg cell. The enucleated egg was placed next to the other cell, then they were shocked to fuse. They were shocked again to start division. The cells were allowed to divide for several days until an early embryonic stage was reached, before being implanted in a surrogate mother.

The first cloned agricultural animal was Dolly, a sheep who was born in 1996. The success rate

of reproductive cloning at the time was very low. Dolly lived for six years and died of a lung tumor. There was speculation that because the cell DNA that gave rise to Dolly came from an older individual, the age of the DNA may have affected her life expectancy. Since Dolly, several species of animals (such as horses, bulls, and goats) have been successfully cloned.

There have been attempts at producing cloned human embryos as sources of embryonic stem cells. In the procedure, the DNA from an adult human is introduced into a human egg cell, which is then stimulated to divide. The technology is similar to the technology that was used to produce Dolly, but the embryo is never implanted into a surrogate mother. The cells produced are called embryonic stem cells because they have the capacity to develop into many different kinds of cells, such as muscle or nerve cells. The stem cells could be used to research and ultimately provide therapeutic applications, such as replacing damaged tissues. The benefit of cloning in this instance is that the cells used to regenerate new tissues would be a perfect match to the donor of the original DNA. For example, a leukemia patient would not require a sibling with a tissue match for a bone-marrow transplant.

When most people think of cloning, they usually think of Dolly the sheep, who in 1996 became the first mammal to be cloned from an adult cell[2]. Unfortunately, Dolly didn't live to a ripe old age, but her birth was an achievement and scientists have learned a lot through the process.

Breeding selected organisms for their desired traits has been going on for thousands of years. More recently, think back to good ol' Gregor Mendel and his peas. His controlled, selective breeding of pea plants was a form of genetic manipulation.

To start, here's a little terminology to make all of this a little easier to understand. A **differentiated** cell has reached its final destination and is specialized. It could be a skin cell, a liver cell, or a stinger cell in a bee. Some differentiated cells can become dedifferentiated and then coaxed in the lab to become a different type of cell. These cells are called **totipotent**.

You've probably heard of **stem cells**, if not through science classes, then through the media. Stem cells are cells that are not differentiated. They have the potential to become any type of cell. That's why they are like the holy grail of genetics.

Embryonic stem cells are **pluripotent**. They can become any type of cell. Adult stem cells cannot become all types of cells, but can give rise to many cell types. For this reason, embryonic stem cells are highly coveted. Their use does not come without difficult ethical issues, as we will discuss later.

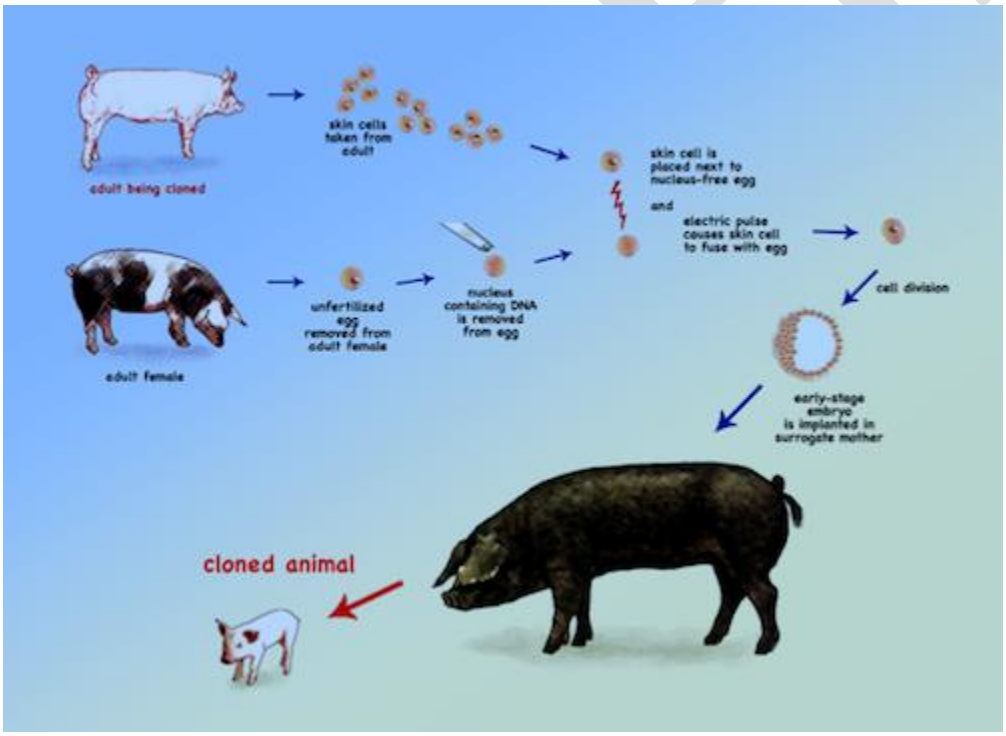
Recently, scientists have learned how to convert differentiated cells into pluripotent cells. These cells are **induced pluripotent cells (iPS)**.

Now that we have the terminology down, on to cloning we go. The first plant to be cloned from a single cell was a carrot. Yep, Bugs Bunny would be proud. Cells were taken from a carrot's roots by Charles Steward and his students in the 1950s and cultured in the lab. These cells eventually produced a plant. This showed that somehow adult plant cells could dedifferentiate and then result in all cell types of the plant. Pretty slick, Dr. Steward.

This method is used by many industries. Think about the lumber industry. What if we discovered a particular tree that was resistant to disease and could grow very tall and thick, and also enjoyed long walks on the beach? This could be a big money maker. We could take a sample and remove individual cells to grow in the lab. These cells can produce seedlings that can be transplanted into the ground. What's the end result of this hard work? Several trees genetically identical to the

original tree.

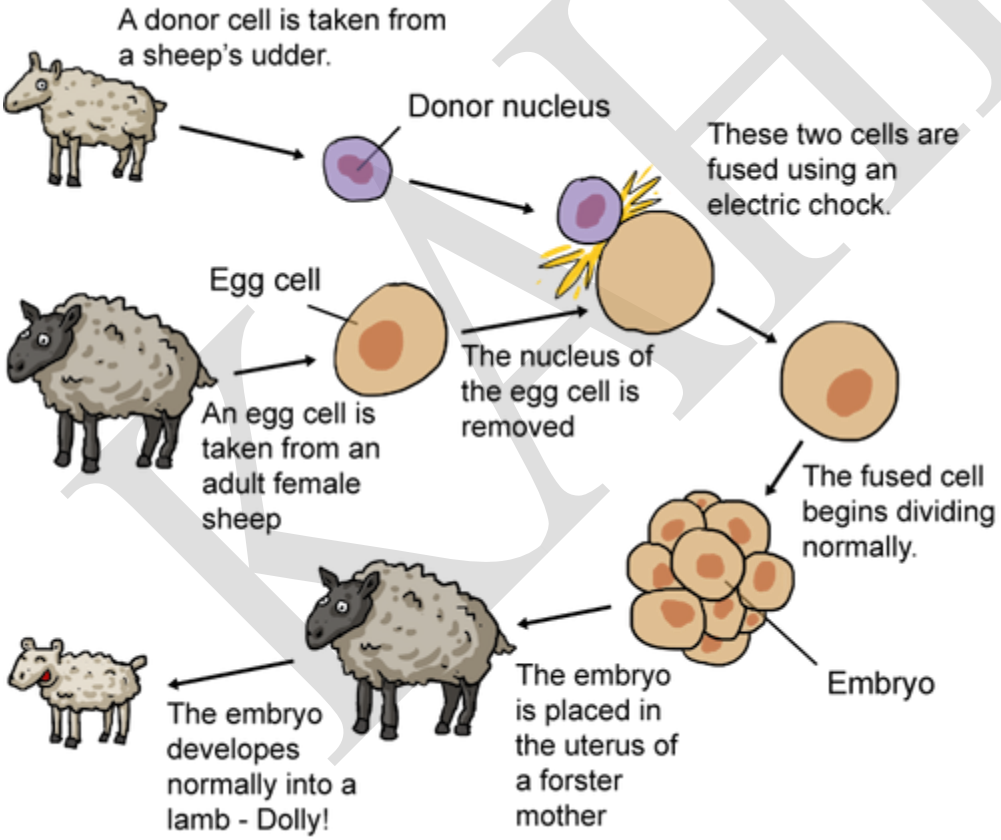
Animal cloning is a little different, and a little more complex. Animals cannot be cloned using the technique for plant cells because usually differentiated animal cells cannot be grown in culture. Instead, scientists use **nuclear transplantion**. Yep, it's just as it sounds. You've heard of heart and kidney transplants. In this case, the nucleus of a differentiated cell is inserted into a fertilized or unfertilized egg cell where the nucleus has been removed (or **enucleated**). The fertilized egg divides several times in the lab and become an embryo. The embryo is then implanted into a surrogate. The resulting animal is genetically identical to the organism whose nucleus was transplanted.



This technique was used to clone Dolly the sheep. Dolly was a super big deal. Scientists did not

know whether or not an already differentiated cell could be used to clone an entire organism. A differentiated cell is already programmed to do its job. Could it direct development from the earliest stages?

The answer was yes, and Dolly was proof. Researchers developed a technique to dedifferentiate cells from six-year-old sheep udder tissue (mammary cells) in the lab. They then transferred these cells to sheep egg cells in which the nuclei had been removed. The cells were allowed to divide in the lab and eventually the embryos were implanted into surrogate female sheep. Dolly was the only live lamb born.

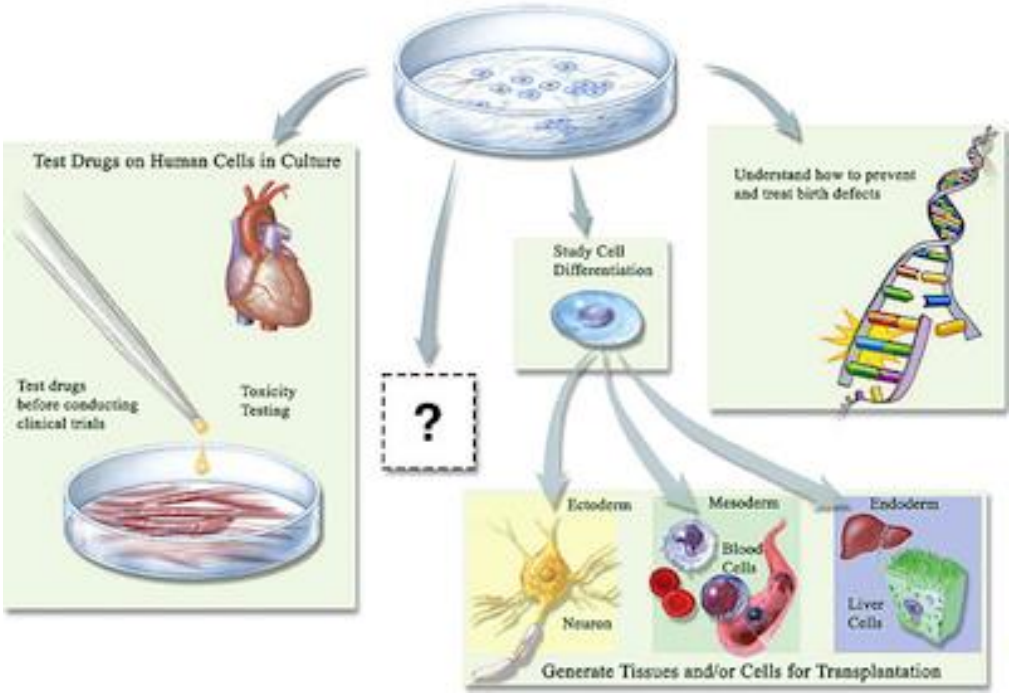


This achievement was monumental, but problems were soon discovered with Dolly. The normal lifespan of sheep is about twelve years. At the age of six, Dolly suffered from conditions

normally seen in much older sheep and was euthanized. Scientists think poor Dolly's health problem could be because the nucleus used to clone Dolly was from a six year old sheep. If that doesn't belong in a sci-fi movie, we don't know what does.

Scientists have noticed that many cloned animals are prone to health issues. A closer look at the transplanted nuclei of several organisms suggests that they are not fully dedifferentiated. This could explain the premature aging and susceptibility to disease shown by cloned animals.

Cloning animals may be beneficial to the agricultural business, but what about human cloning? Scientists aren't as interested in creating another human being as generating stem cells from human embryos. Remember, embryonic stem cells can become any type of cell. They are not differentiated yet. These cells can be studied to better understand how a cell becomes differentiated. It is thought that they hold great promise for the treatment of medical conditions.



Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a protein produced by a jellyfish *Aequorea victoria*; which produces glowing points of light around the margin of it's umbrella. The light arises from yellow tissue masses that each consist of about 6000-7000 photogenic cells. These cells generate light by a process of bioluminescence, whose components include a calcium-activated photoprotein (aequorin) that emits blue-green light and an accessory green fluorescent protein (GFP), which accepts energy from aequorin and re-emits it as green light. GFP a 238 amino acids protein which is very stable in neutral buffers up to 65oC, and displays a broad range of pH stability from 5.5 to 12. The protein is intensely fluorescent, with a quantum efficiency of approximately 80% and molar extinction coefficient of 2.2 x 104 cm-1 M-1. GFP fluoresces maximally when excited at 400nm with a lesser peak at 475nm, and fluorescence emission peaks at 509nm. The intrinsic fluorescence of the protein is due to a unique covalently attached chromophore, which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly. The gene for GFP has been isolated and has become a useful tool for making expressed proteins fluorescent by creating chimeric genes composed of those of GFP and its different colour variants linked to genes of proteins of interest. Making it possible to have an *in vivo* fluorescent protein, which may be followed in a living system.

- There have been several recent developments for the use of GFP and it's colour variants. Wild type GFP has two excitation peaks, a major one at 395nm (long wave UV, causes rapid quenching of the fluorescence) and a smaller one at 475nm (blue) and an emission peak at 509nm (green). For general fluorescence microscopy purposes, investigators have been using normal FITC filter sets for viewing GFP. These are inadequate for wild type GFP both in excitation 475-495nm, and emission 520-560nm. To alleviate this problem, several modified versions of GFP were constructed which have increased fluorescence (serine to threonine substitution at position 65 increased fluorescence 5-6 times), but perhaps more important, the major excitation peak has been red-shifted to 490nm with the emission staying at 509nm. This is better for use of FITC filter sets as this modified

GFP has the same excitation range as FITC. Furthermore, in confocal microscopy the main laser line used for GFP excitation is from the argon laser at 488nm, there is no good commonly used laser line near 395nm. In Arabidopsis plants and cells, poor or no fluorescence was seen when transform with gfp cDNA because the expression of GFP was curtailed by aberrant mRNA splicing. Therefore, modified forms of GFP were created to restore and improve expression of the fluorescent protein. The modified gene now contains an altered codon to remove a cryptic plant intron. Since then, other modifications have given further improvements in the brightness of the emission and different colour variants of GFP have been produced e.g. in order from shortest to longest emission spectra: blue (FP or BFP), cyan (CFP), green (GFP), yellow (YFP) and red (RFP). This now makes it possible to make double-labelled specimens expressing two or more fluorescently labelled proteins. Added peptide sequences also allow targeting of GFP intracellular organelles like the lumen of the endoplasmic reticulum.

Improving GFP

GFP is amazingly useful for studying living cells, and scientists are making it even more useful. They are engineering GFP molecules that fluoresce different colors. Scientists can now make blue fluorescent proteins, and yellow fluorescent proteins, and a host of others. The trick is to make small mutations that change the stability of the chromophore. Thousands of different variants have been tried, and you can find several successes in the PDB. Scientists are also using GFP to create biosensors: molecular machines that sense the levels of ions or pH, and then report the results by fluorescing in characteristic ways. The blue fluorescent protein that has been modified to sense the level of zinc ions. When zinc, shown here in red, binds to the modified chromophore, shown here it bright blue, the protein fluoresces twice as brightly, creating a visible signal that is easily detected.

Engineering GFP

The uses of GFP are also expanding into the world of art and commerce. Artist Eduardo Kac has created a fluorescent green rabbit by engineering GFP into its cells. Breeders are exploring GFP as a way to create unique fluorescent plants and fishes. GFP has been added to rats, mice, frogs, flies, worms, and countless other living things. Of course, these engineered plants and animals are still controversial, and are spurring important dialogue on the safety and morality of genetic engineering.

Possible Questions

2 Marks

Define cell transformation.

What are the different methods of physical cell transformation?

What is green fluorescent protein.

Define gene therapy.

8 Marks

Explain different practices of cell transformation.

Describe manipulation of genes through molecular techniques

Explain cell and organism cloning

What is green fluorescent protein and explain its application.

Describe gene therapy and its application.

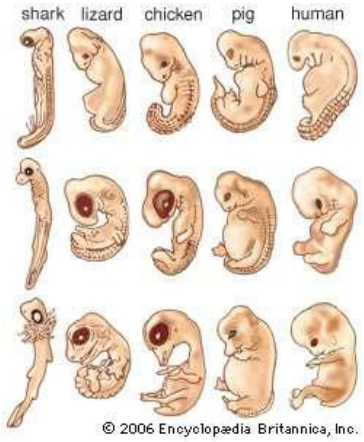
UNIT -IV

SYLLABUS

Embryology: Collection and preservation of embryos; culturing of embryos; gametogenesis and fertilization in animals; types of cleavage pattern; role of maternal contributions in early embryonic development; *In vitro* fertilization and stem cell research.

Embryology

Embryology, the study of the formation and development of an embryo and fetus. Before widespread use of the microscope and the advent of cellular biology in the 19th century, embryology was based on descriptive and comparative studies. From the time of the Greek philosopher Aristotle it was debated whether the embryo was a preformed, miniature individual (a homunculus) or an undifferentiated form that gradually became specialized. Supporters of the latter theory included Aristotle; the English physician William Harvey, who labeled the theory epigenesis; the German physician Caspar Friedrick Wolff; and the Prussian-Estonian scientist Karl Ernst, Ritter von Baer, who proved epigenesis with his discovery of the mammalian ovum (egg) in 1827. Other pioneers were the French scientists Pierre Belon and Marie-François-Xavier Bichat.



vertebrate embryos: Vertebrate embryos shown during successive stages of development.

Baer, who helped popularize Christian Heinrich Pander's 1817 discovery of primary germ layers, laid the foundations of modern comparative embryology in his landmark two-volume work Über Entwickelungsgeschichte der Thiere (1828–37; "On the Development of Animals"). Another formative publication was A Treatise on Comparative Embryology (1880–91) by the British zoologist Frances Maitland Balfour. Further research on embryonic development was conducted by the German anatomists Martin H. Rathke and Wilhelm Roux and also by the American scientist Thomas Hunt Morgan. Roux, noted for his pioneering studies on frog eggs (beginning in 1885), became the founder of experimental embryology. The principle of embryonic induction was studied by the German embryologists Hans Adolf Eduard Driesch, who furthered Roux's research on frog eggs in the 1890s, and Hans Spemann, who was awarded a Nobel Prize in 1935. Ross G. Harrison was an American biologist noted for his work on tissue culture.

Human embryogenesis

Embryogenesis, the first eight weeks of development after fertilization, is an incredibly complicated process. It's amazing that in eight weeks we're transforming from a single cell to an organism with a multi-level body plan. The circulatory, excretory, and neurologic systems all begin to develop during this stage. Luckily, like with many complex biological concepts, fertilization can be broken down into smaller, simpler ideas. The big idea of embryogenesis is going from a single cell to a ball of cells to a set of tubes.

Very Beginning

- **Step 1**: a *zygote* is the single cell formed when an egg and a sperm cell fuse; the fusion is known as fertilization
- **Step 2**: the first 12-to 24-hours after a zygote is formed are spent in *cleavage* – very rapid cell division

The zygote's first priority is dividing to make lots of new cells, so it's first few days are spent in rapid mitotic division. With each round of division, it doubles in cell number, so the cell number

is increasing at an exponential rate! This division is taking place so quickly that the cells don't have time to grow, so the 32 cell stage known as the *morula* is the same size as the zygote. At this point, the zona pellucida (a protective membrane of glycoproteins that had surrounded the egg cell) is still intact, which also limits how big it can grow.

Blastulation and Cell Differentiation

- **Step 3**: during *blastulation*, the mass of cells forms a hollow ball
- **Step 4**: cells begin to differentiate, and form cavities

Around day 4, cells continue to divide, but they also begin to differentiate and develop more specific forms and functions. When a cell differentiates, it moves down a certain path toward being a specific type of cell (e.g. an ear cell or a kidney cell), and this process (99% of the time) only goes in one direction. Two layers develop: an outer shell layer known as the *trophoblast*, and an inner collection of cells called the inner cell mass. Rather than being arranged in a solid sphere of cells, the *inner cell mass* is pushed off to one side of the sphere formed by the trophoblast. The rest of the fluid-filled cavity is called the *blastocoel*, and the whole setup resembles a snow globe. The outer trophoblast will develop into structures that help the growing embryo implant in the mother's uterus. The inner cell mass will continue to differentiate and parts of it will eventually become the embryo, so it is sometimes called the *embryoblast* (the suffix "blast" means "to make"). This is also the time when the zona pellucida begins to disappear, allowing the ball of cells, now called a *blastocyst*, to grow and change shape. In non-mammal animals, the term for this stage is *"blastula"*, but we will stick with terms that apply to human development for the purposes of this discussion.

At this point, cells in the inner cell mass are *pluripotent*, meaning they can eventually turn into the cells of any body tissue (muscle, brain, bone, etc). During the second week, these cells differentiate further into the *epiblast* and the *hypoblast*, which are the two layers of the *bilaminar disc*. This disc is a flat slice across the developing sphere, and splits the environment into two cavities. The hypoblast is the layer facing the blastocoel, while the epiblast is on the other side. Let's imagine each of these layers as a flat balloon. The balloons expand to fill the space, and

become the two new cavities: the *primitive yolk sac* on the side of the hypoblast and the *amniotic cavity* on the side of epiblast. The amniotic cavity will eventually surround the fetus.

Quick recap: the outermost layer of the sphere is the trophoblast. Inside the sphere are two spaces that are each lined by either the hypoblast or the epiblast. The point where the epiblast and hypoblast press up against each other is known as the bilaminar disc, and this disk is what splits the sphere to make the two cavities.

The hypoblast does not contribute to the embryo, so we will now turn our focus solely on the epiblast.

Making Tubes

- **Step 5**: During *gastrulation* the three germ layers form; the cell mass is now known as a gastrula
- **Step 5a**: The *primitive streak* forms
- **Step 6**: The *notochord* is formed

Week 3 of development is the week of *gastrulation*. A germ layer is a layer of cells that will go on to form one of our organizational tubes. Our anatomy can really be boiled down to an inner tube (our digestive tract), and a series of tubes that wrap around it. The three germ layers that will translate into these tubes are the *ectoderm*, the *mesoderm*, and the *endoderm*.

Germ Layer	**What does the prefix mean?**	**Goes on to form:**
Ectoderm	Outer, external	Epidermis (outer layer of skin), hair, nails, brain, spinal cord, peripheral nervous system
Mesoderm	Middle	Muscle, bone, connective tissue, notochord, kidney, gonads, circulatory system
Endoderm	Within	Epithelial lining of the digestive tract; Stomach, colon, liver, pancreas, bladder, lung

The first step of gastrulation is the formation of the primitive streak (~ day 16). Let's imagine the bilaminar disc as two tier cake. Imagine taking a knife and cutting into just the top layer (the epiblast) like you're going to cut a slice.

Two tier cake representing epiblast and hypoblast with cut through epiblast layer

This cut is the primitive streak, and it cuts from the caudal (anus) end in toward the end that will eventually become the head (the rostral end). This streak determines the midline of the body, and separates the left and right sides. Like all deuterostomes, humans have bilateral symmetry, which means that there is a single across which we can split ourselves to make mirror images. What we are actually seeing when we look at a primitive streak are moving cells. They are going from the epiblast and moving down so they end up between the original epiblast layer and the hypoblast. I've always imagined the motion like water falling down a waterfall. The first layer to invaginate dives the deepest and ends up closest to the hypoblast – this is the endoderm. The next layers will become the mesoderm, and the cells of the epiblast that continue to border the amniotic cavity are the ectoderm. We now have three germ layers, all of which will contribute to the developing embryo. In the picture below, the anus end is facing us.

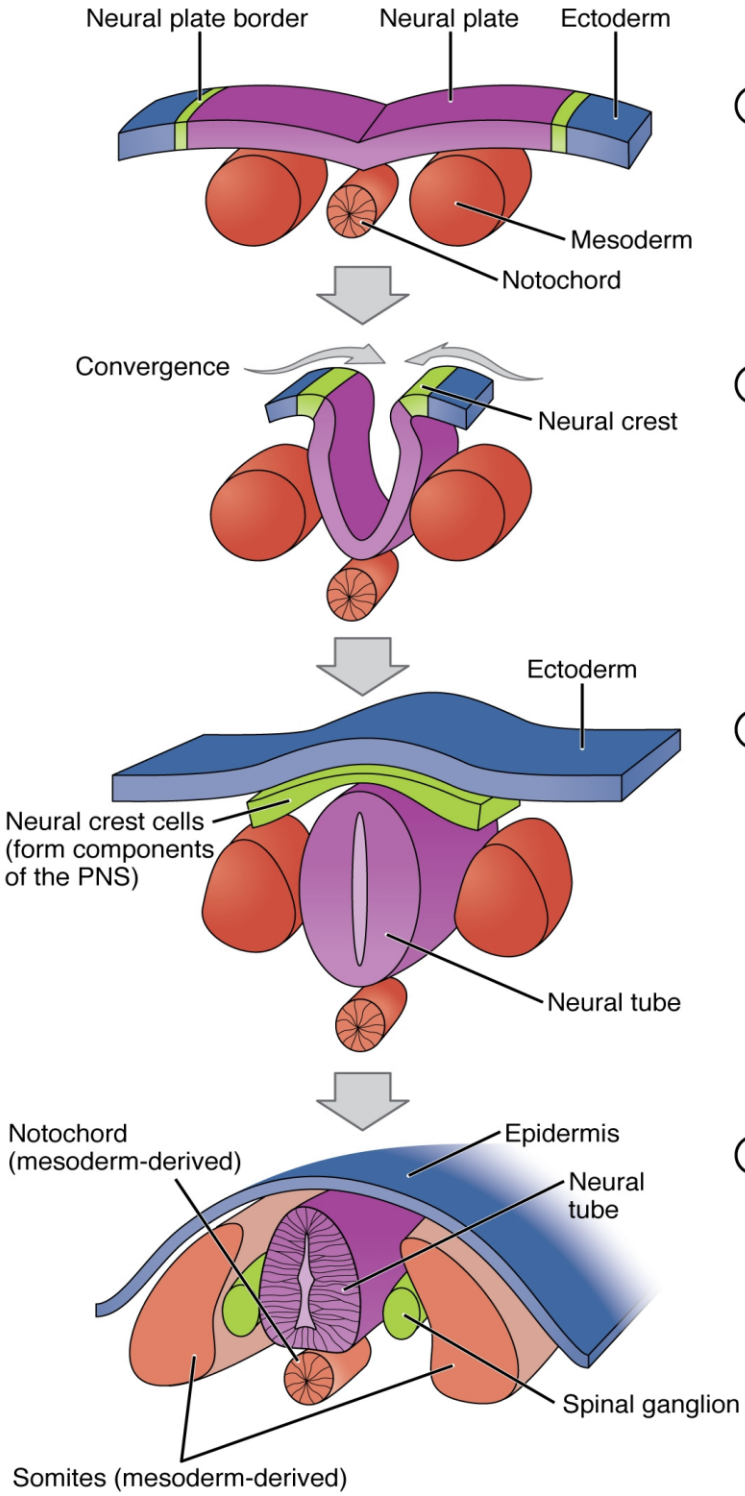
Directly beneath the primitive streak the mesoderm (the middle germ layer) forms a thin rod of cells known as the notochord. The notochord helps define the major axis of our bodies, and is important in inducing the next step of embryogenesis, when we finally start to make our tubes! The notochord is a defining feature of the Chordate phylum, and will eventually become our intervertebral discs.

Neurulation

- **Step 6**: Tubes form, making a *neurula*
- **Step 6a**: The notochord induces the formation of the *neural plate*
- **Step 6b**: The neural plate folds in on itself to make the *neural tube* and *neural crest*
- **Step 7**: The mesoderm has five distinct categories

All this and we still haven't made tubes! Now that we have successfully made the cell layers, we have to create the final 3D product. The first step in this rolling is the creation of the notochord. The notochord causes the ectoderm above it to form a thick flat plate of cells called the neural plate. The neural plate extends the length of the rostral-caudal axis. The neural plate then bends back on itself and seals itself into a tube known as the neural tube that fits underneath the ectoderm. The borders of where the neural plate had been get pulled under with it, and become the neural crest. The neural tube will become the brain and spinal cord.

The neural crest is sometimes called the fourth germ layer, because the cells that become the sympathetic and parasympathetic nervous systems, melanocytes, Schwann cells, even some of the bones and connective tissue of the face.



1. Neuroectodermal tissues differentiate from the ectoderm and thicken into the neural plate. The neural plate border separates the ectoderm from the neural plate.

2. The neural plate bends dorsally, with the two ends eventually joining at the neural plate borders, which are now referred to as the neural crest.

3. The closure of the neural tube disconnects the neural crest from the epidermis. Neural crest cells differentiate to form most of the peripheral nervous system.

4. The notochord degenerates and only persists as the nucleus pulposus of the intervertebral discs. Other mesoderm cells differentiate into the somites, the precursors of the axial skeleton and skeletal muscle.

Illustration of neurulation process

Meanwhile, the mesoderm can be subdivided into the axial, paraxial, intermediate, and lateral plate mesoderms. The notochord came from the axial mesoderm. The paraxial mesoderm will give rise to somites, which will differentiate into muscle, cartilage, bone, and dermis. Somite derivatives create a segmented body plan (see right). The intermediate mesoderm is the origin of our urogenital system – our kidneys, gonads, adrenal glands, and the ducts that connect them. The lateral plate mesoderm will give rise to the heart (the first organ to develop!), blood vessels, the body wall, and the muscle in our organs.

Also at the same time, the endoderm is rolling into a tube as well – the digestive tract. The digestive tract is subdivided into the foregut, midgut, and hindgut. Each subdivision has its own nerve and blood supply. Organs related to the GI tract actually start off as outpouchings of this tube. The foregut gives rise to the esophagus, stomach, part of the duodenum, and the respiratory bud, which will eventually develop into the lungs. The second half of the duodenum through to the transverse colon arise from the midgut. The remainder of the GI tract, including the rest of the transverse colon, the descending colon, the sigmoid colon, and the rectum are formed from the hindgut.

That's what is going in with each of the three layers. While this is happening, the mesodermal layers are circling around the endoderm, and the part of the ectoderm that will become the skin is circling around both of the other layers. Some tubes, likes the neural tube, are closing, while the gut tube is connecting to the ectoderm to form the mouth and the anus. By the time eight weeks have passed, all of our tubes are in order, the primitive heart has been beating for almost five weeks, and development is well on its way!

Consider the following

The gut tube is the only developmental tube that is supposed to remain an open cylinder. If the neural tube does not close, it creates a life-threatening condition known as spina bifida. Spina bifida can occur due to genetic factors, but may also be caused by a lack of folic acid during pregnancy or if the mother has uncontrolled diabetes. Spina bifida can lead to weakness and paralysis of the legs, bladder and bowel control issues, and other physical problems. Children

with spina bifida often struggle academically, potentially due to problems in the development of the central nervous system. While there is no known cure for spina bifida, the introduction of folic acid into everyday foods like cereal and bread has drastically reduced the incidence of neural tube defects in newborns.

Culturing embryos in the laboratory

In vitro fertilisation (IVF)

Depending on a woman's age, anywhere between 1 and 30 follicles, known as 'recruits', will begin to develop in each menstrual cycle. Whatever her age, though, only one of these developing follicles will dominate and ovulate at the level of follicle stimulating hormone (FSH) that a woman produces naturally.

With in vitro fertilisation (IVF), the goal is to keep the level of FSH constant, and thus encourage more of the recruits to grow and to develop mature eggs, which are collected surgically under vaginal ultrasound guidance. The eggs are then fertilised in the laboratory, cultured for several days, and then one embryo (or rarely two) is transferred back into the woman's uterus. If there are additional embryos, they may be frozen and stored for later use if of suitable quality.

IVF cycles include a preparation month which involves a nursing and possible counselling appointment followed by these steps:

1. Stimulating the ovaries with injections of FSH
2. Preventing premature ovulation (the luteinising hormone (LH) surge) by shutting down communication between the brain and the ovaries, so that the eggs are not lost before they can be collected
3. 'Triggering' ovulation by replacing the LH surge at mid cycle with an injection of human chorionic gonadotrophin (hCG)
4. Collecting the eggs and sperm
5. Culturing embryos in the laboratory

6. Transferring the embryo/s
7. Supporting the endometrium in the luteal phase with hCG or progesterone.

Process of Gametogenesis in Animals

The series of changes through which the germinal epithelial cells pass to produce germ cells or gametes is called gametogenesis (Fig. 11). The development of eggs is oogenesis and that of the sperm is spermatogenesis. The phases of development in both the gametes are essentially similar.

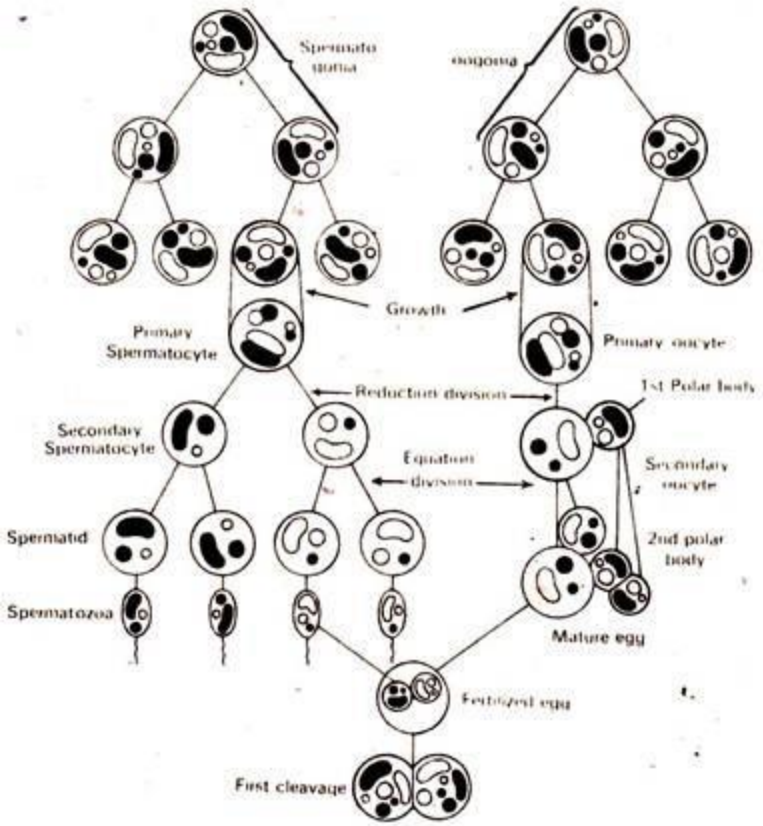


Fig. 11. Gametogenesis

Spermatogenesis:

1. It occurs in the testes. The walls of the seminiferous tubules of vertebrates contain primordial germ cells.

2. The primordial germ cells begin to enlarge with increased metabolism and undergo mitotic division, and produce spermatogonia.

3. The spermatogonium divides meiotically (first meiosis) resulting in two spermatocytes.

4. Due to second meiotic division (mitosis) of spermatocyte four haploid-spermatids are formed.

5. Spermatids metamorphose to spermatozoa. This is spermiogenesis.

(a) In this process spermatid increases in size; centriole divides into two; distal centriole forms the main axis of the tail the axial filament is surrounded by a fibre coat; mitochondria form a spiral sheath; golgi complex forms acroblast and give rise to acrosome.

6. The mature spermatozoon has three distinct zones head, trunk and tail. It can move actively in fluid.

Oogenesis:

1. It occurs in the ovary. The phases of division in multiplication are similar to those in spermatogenesis.

2. The primary germ cells of the ovary undergo several mitotic divisions to produce oogonia.

3. The oogonium gives rise to primary oocytes mitotically.

4. A primary oocyte undergoes first meiotic division and produces one large secondary oocyte and one small first polar body or polocyte.

5. The oocyte and the polocyte divide mitotically (second meiotic division). The oocyte produces one large ootid and one small second polar body and the polocyte two polar bodies.

6. In oogenesis one haploid ootid and three polar bodies are formed. On maturity, the ootid is transformed into ovum.

Fertilization:

Fertilization is complete fusion of sperm and ovum except the chromosomes, resulting in a single diploid cell, the Zygote. The union of the nuclei of male and female gametes is karyogamy and that of their cytoplasm isplasmogamy.

The steps in fertilization are:

I. Approach of the spermatozoon to the egg:

(a) By chemotactic movement the sperm swims towards the egg.

(b) The jelly coat of the egg produces fertilizin and the sperms produce antifertilizin.

(c) The fertilizin and the antifertilizin combine to form an initial bond to facilitate penetration of spermatozoon into the egg.

II. Penetration of the sperm:

(a) The bound or agglutinated spermatozoon produces an enzyme, lysine, which dissolves egg membranes in the local area and clear the path for spermatozoon to reach the egg surface.

(b) The acrosomal filament is pushed through the jelly and vitelline membrane, and touches the surface membrane of the egg cytoplasm.

III. Reaction in egg during sperm penetration:

(a) Coming in contact with the acrosomal filament the egg cytoplasm bulges forward to produce a conical projection, the fertilization cone.

(b) It gradually engulfs the spermatozoon and carries it inward.

(c) Simultaneously profound cortical reaction occurs in the cytoplasm.

(d) Immediately after penetration of the spermatozoon the vitelline membrane separates from the plasma membrane. The vitelline membrane thickens and forms the fertilization membrane.

(e) The cortical granules swell, become liquified and the fluid is released in the space between egg cytoplasm and fertilization membrane.

IV. Behaviour of sperm within the ovum:

(a) The head and middle piece enter the egg cytoplasm but the tail is left outside.

(b) The egg completes its meiosis immediately and loses the centriole.

(c) The sperm rotates 180° and its middle part comes to the front end. The path along which the sperm moves within the egg is known as fertilization path.

(d) The egg pronucleus moves to the periphery and the movement of the sperm is oriented to it. This is copulation path.

(e) The sperm pronucleus swells up. The centrosome and the centriole in the middle piece form aster around the two pronuclei.

(f) The sperm centriole divides into two, move to the poles being connected by fibres.

(g) The nuclear membranes disappear, the chromosomes of the male and female pronuclei are arranged in the equator of the spindle.

(h) It is similar to metaphase plate of meiosis and immediately followed by the cleavage of the zygote.

Importance of Fertilization:

(a) The sperm entry activates the secondary oocyte to complete its maturation division.

(b) Restores diploid number of chromosomes and recombines the maternal and paternal genetic materials.

(c) Evokes development and cleavage of egg to form a complete organism.

Gametogenesis and Fertilization | Animals

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6. The mature spermatozoon has three distinct zones—head, trunk and tail. It can move actively in fluid.

Structure of Human Spermatozoa:

In human, the spermatozoon (Fig. 40.2) measures 60 µm long and 2.5 to 3.5 µm in diameter at the broadest point in the head. The spermatozoon is rich in deoxyribonucleic acid (DNA).

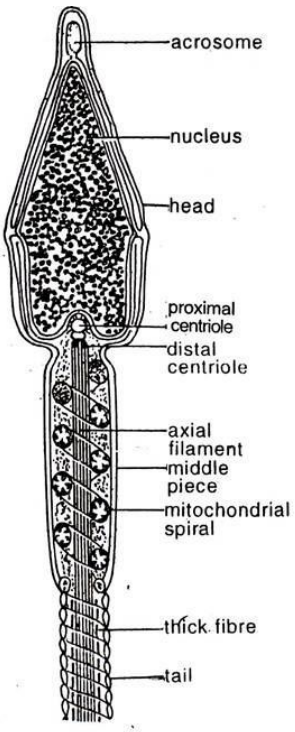

Fig. 40.2. A human sperm

Head:

The head is constituted by an anterior acrosome (galea capitis) and a nucleus.

a. Acrosome:

Acrosome is a double-walled, granulated sac, convex anteriorly and flat posteriorly. It contains enzymes to dissolve the zona pellucida (egg membrane) of the ovum to facilitate entry of the sperm for fertilization.

b. Nucleus:

It is a dense structure, rich in DNA, narrower anteriorly and bears a depression at the middle of the broad posterior end.

Middle piece:

It connects the head with the tail piece. The proximal centriole of the anterior end fits into the depression of the head and the distal centriole is attached with the nine axial filaments in the mid piece. Elongated mitochondria form a firmly coiled spiral around the axial filaments. The middle piece provides energy for locomotion of sperms.

Tail piece:

It has two portions:

a. The broad anterior main piece and

b. A narrow, posterior terminal end piece.

a. The main piece of the tail consists of a spiral mitochondrial sheath surrounding a group of eleven fibrils. Two fibrils are centrally placed and the rest form a ring around them. Contraction and relaxation of peripheral fibrils help the spermatozoa to move forward. The enzyme adenosine triphosphatase (ATPase) is present in the whole length of the main piece.

b. Terminal end piece (flagella). It is the slender, last portion of the tail piece. The fibrils are naked and resemble cilia.

Oogenesis:

1. It occurs in the ovary. The phases of division in multiplication are similar to those in spermatogenesis.

2. The primary germ cells of the ovary undergo several mitotic divisions to produce oogonia.

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5. The oocyte and the polocyte divide mitotically (second meiotic division). The oocyte produces one large ootid and one small second polar body and the polocyte two polar bodies.

6. In oogenesis one haploid ootid and three polar bodies are formed. On maturity, the ootid is transformed into ovum. The ova are of one type only. The nucleus with haploid chromosome number is termed pronuclear.

Structure of Ova:

The ovum is a large, round structure. In human, according to the stage of development, it measures 117 to 142 µm in diameter. The cytoplasm of the ovum is termed yolk or ooplasm, the nucleus as the germinal vesicle and the nucleolus as germinal spot.

The yolk comprises:

(a) Cytoplasm similar to that of other cells and

(b) Deutoplasm or nutritive yolk, consists of fatty droplets containing lecithin, a phospholipid.

The mode of distribution of deutoplasm in the ovum differ in different animals. During oogenesis mitochondria increase in numbers and become uniformly distributed in the cytoplasm. The Golgi apparatus spreads out, being restricted usually near the periphery. The amount of yolk in the ovum varies in different groups of animals.

The mature ovum or egg is surrounded by two membranes, the plasma membrane (egg membrane) and the vitelline membrane. The vitelline membrane is thin and surrounds the plasma membrane. In addition to these primary membranes, secondary membrane secreted by the ovary and tertiary membrane secreted by the glands in the oviduct may surround the egg.

In branchiostoma, only the plasma membrane and vitelline membrane are present. In frogs and toads a sphere of jelly around the egg is enclosed in a thin membrane. In birds, a two-layered shell membrane and a calcareous shell are secreted by the oviduct and, in mammals, the additional layers— zona pellucida and chorona radiata are possibly primary and secondary membrane, respectively, (Fig. 40.3).

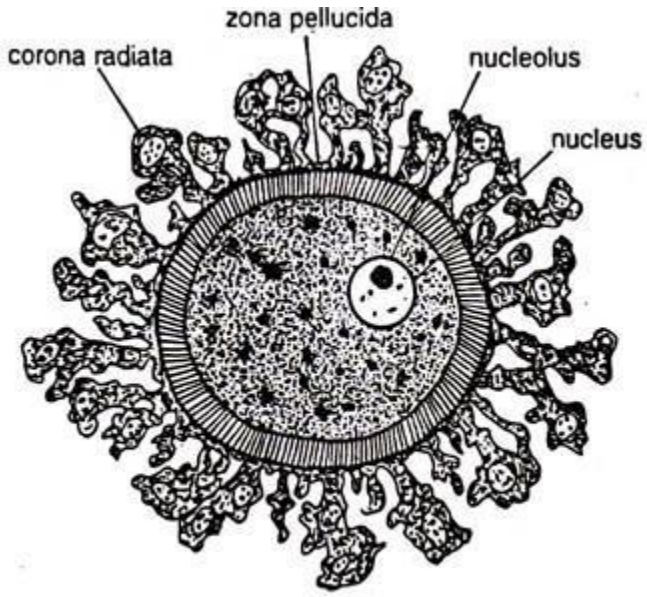

Fig. 40.3. A human ovum

Types of Eggs:

The eggs are classified on the amount and position of deutoplasm (yolk) present in them.

Homolecithal or Isolecithal egg:

The amount of yolk is small and present chiefly in droplets and minute spherules, uniformly distributed in the cytoplasm. True homolecithal eggs are found in eutherian mammals.

Microlecithal egg:

The amount of yolk is small.

Examples: Cnidarians.

Megalecithal egg:

The amount of yolk is large.

Examples: Some invertebrates, reptiles, birds and prototherian mammals.

Centrolecithal egg:

The yolk is present around the nucleus in the form of a large sphere.

Example: Arthropod eggs.

Telolecithal egg:

Eggs are large, megalecithal and the yolk occupies almost the entire egg, except a minute area in the animal pole. In chordates, telolecithality occurs in varying degrees.

a. In Branchiostoma, the yolk is concentrated at one pole, the region of future endoderm cells.

b. In cyclostomes and amphibians, the quite high amount of yolk is concentrated in the vegetal pole.

c. In reptiles and birds, the amount of yolk is large and occupies almost the entire egg and the active cytoplasm. The egg nucleus forms a small cap on the yolk in the animal pole.

Fertilization:

Fertilization is a complete fusion of sperm and ovum except the chromosomes, resulting in a single diploid cell, the zygote. The union of the nuclei of male and female gametes is karyogamy and that of their cytoplasm is plasmogamy.

The steps in fertilization (Fig. 40.4) are:

I. Approach of the spermatozoon to the egg:

a. By chemotactic movement the sperm swims towards the egg.

b. The jelly coat of the egg produces fertilizin and the sperms produce antifertilizin.

c. The fertilizin and the antifertilizin combine to form an initial bond to facilitate penetration of spermatozoon into the egg.

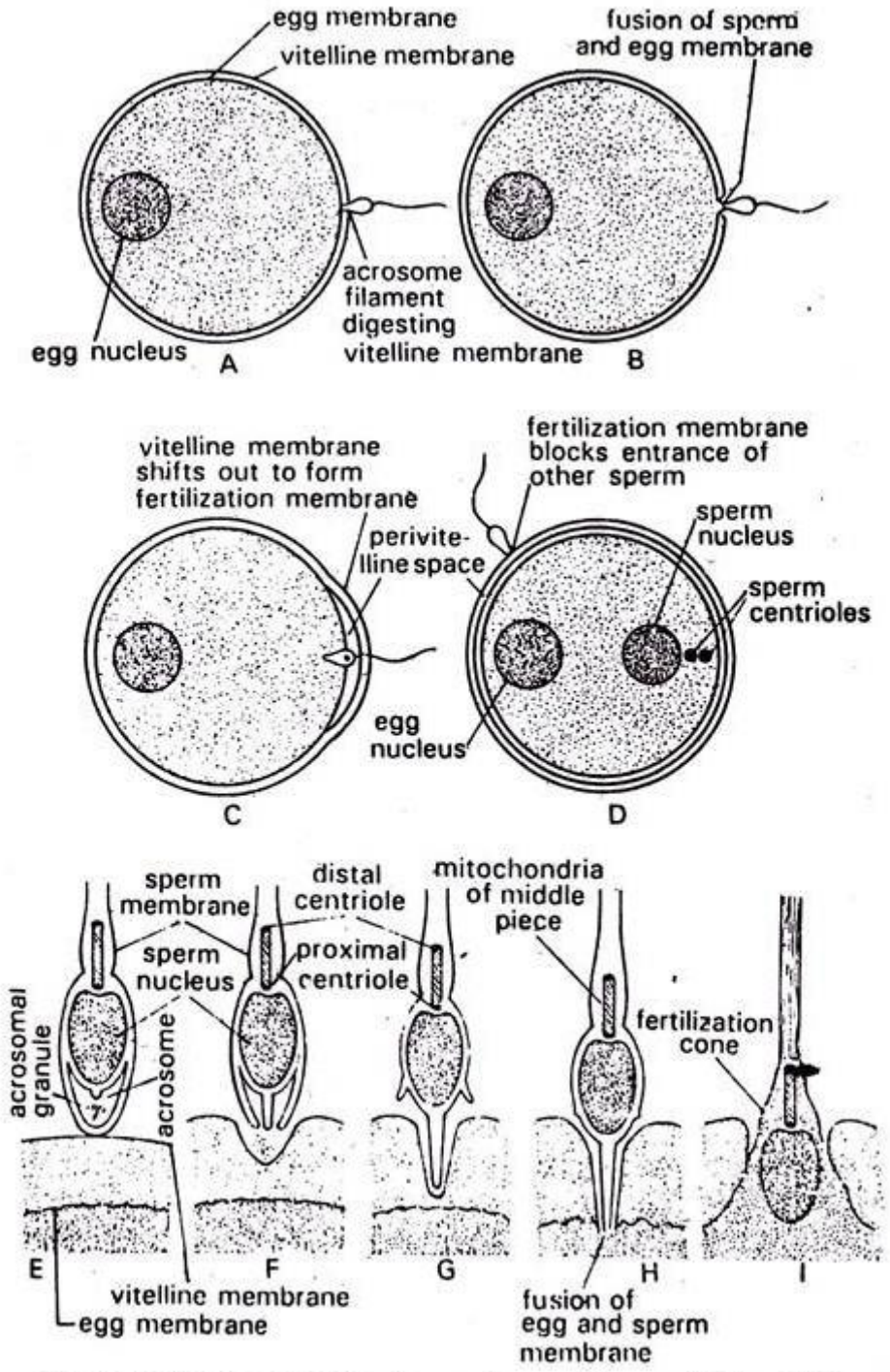

Fig. 40.4. Fertilization. A-D. Union of a spermatozoon and an ovum. E-I. Insemination

II. Penetration of the sperm:

a. The bound or agglutinated spermatozoon produces an enzyme, lysine, which dissolves egg membranes in the local area and clears the path for spermatozoon to reach the egg surface.

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a. Coming in contact with the acrosomal filament the egg cytoplasm bulges forward to produce a conical projection, the fertilization cone.

b. It gradually engulfs the spermatozoon and carries it inward.

c. Simultaneously profound cortical reaction occurs in the cytoplasm.

d. Immediately after penetration of the spermatozoon, the vitelline membrane separates from the plasma membrane. The vitelline membrane thickens and forms the fertilization membrane which prevents entry of other sperms and thus ensures entrance of only one sperm into the egg.

e. The cortical granules swell, become liquefied and the fluid is released in the space between egg cytoplasm and fertilization membrane.

IV. Behaviour of sperm within the ovum:

a. The head and middle piece enter the egg cytoplasm but the tail is left outside.

b. The egg completes its meiosis immediately and loses the centriole.

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b. Restores diploid number of chromosomes and recombines the maternal and paternal genetic materials.

c. Evokes development and cleavage of egg to form a complete organism.

Cleavage: Meaning, Planes and Types | Embryology

1. Meaning of Cleavage:

Fertilization results into the formation of zygote. The process of segmentation (cleavage) immediately follows fertilization or any other process which activates the egg. Cleavage consists of division of the zygote into a large number of cellular entities. The cells which are produced during segmentation are called blastomeres.

a. Meridional plane of cleavage:

When a furrow bisect both the poles of the egg passing through the median axis or centre of egg it is called meridional plane of cleavage. The median axis runs between the centre of animal pole and vegetal pole.

b. Vertical plane of cleavage:

When a furrow passes in any direction (does not pass through the median axis) from the animal pole towards the opposite pole.

c. Equatorial plane of cleavage:

This type of cleavage plane divides the egg halfway between the animal and vegetal poles and the line of division runs at right angle to the median axis.

d. Latitudinal plane of cleavage:

This is almost similar to the equatorial plane of cleavage, but the furrow runs through the cytoplasm on either side of the equatorial plane.

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3. Types of Cleavage:

Considerable amount of reorganisation occurs during the period of cleavage and the types of cleavage depend largely upon the cytoplasmic contents.

Different types of cleavage encountered in different eggs are catalogued below:

a. Holoblastic op total cleavage:

When the cleavage furrows divide the entire egg.

It may be:

Equal:

When the cleavage furrow cuts the egg into two equal cells. It may be radially symmetrical, bilaterally, symmetrical, spirally symmetrical or irregular.

Unequal:

When the resultant blastomeres become unequal ir size.

b. Meroblastic cleavage:

When segmentation takes place only in a small portion of the egg resulting in the formation of blastoderm, it is called meroblastic cleavage. Usually the blastoderm is present in the animal pole and the vegetal pole becomes laden with yolk which remains in an tihcleaved state, i.e., the plane of division does not reach the periphery of blastoderm or blastodisc.

c. Transitional cleavage:

In many eggs, the cleavage is atypical which is neither typically holoblastic nor meroblastic, but assumes a transitional stage between the two.

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4. Effects of Yolk in Cleavage:

The fertilized egg in most cases contains yolk, which are inert bodies. During division these bodies exert mechanical influences. In the egg of Amphioxus, the yolk is thin and remains uniformly distributed. Therefore the division is complete and early divisions occur at a very quicker rate.

The amphibian egg contains yolk which is localised at the vegetal pole. Here division initiates from the animal pole and extends towards the vegetal pole, where the progress of cleavage slows down considerably.

Consequently, the animal pole divides faster than the vegetal pole. The eggs of reptiles and birds are fully laden with large masses of yolk, thus restricting the cytoplasm and nucleus on the periphery as a circular disc on the animal pole. Here the lines of cleavage divide only the small animal pole region. Such effects of yolk on cleavage pattern influence the pattern of further development.

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5. Mechanism of Cleavage:

The incidence of cleavage provides unique opportunity to study the mechanism of cell division and specially the role of different cell organelles during division.

Opinions differ regarding the accumulation of force for the initiation of cleavage and following factors are believed to be responsible for controlling the cleavages:

(a) Localised expansion of cortex.

(b) Increased stiffness of the cortical cytoplasm.

(c) Increase of tangential force activity in the cortex.

(d) Contractile nature of the regions near the cortex and

(e) Formation of new cell membrane from the subcortical cytoplasm.

Though the abovementioned factors are not clearly understood, it is evident that three structures present within the cell: Cortical layer, Spindle structures and Chromosomes play the important part.

The energy which is required during the process is supplied by the metabolic activity of the developing egg. Besides the factors involved in segmentation, there are cleavage laws which govern the behaviour of the cells during cleavage.

Sach's rules:

The blastomeres tend to divide into identical daughter cells and a cleavage furrow tends to cut the previous cell at right angles.

Hart wig's laws:

The position of nucleus is vital and it tends to lie at the centre of the protoplasmic content of the cell. The nucleus exerts influence on cleavage. The long axis of mitotic spindle usually coincides with the long axis of the protoplasmic content. During cleavage the long axis of the protoplasm has the tendency to cut transversely.

Balfour's law:

The rate of cleavage is inversely proportional to the amount of yolk material present in the egg.

---

6. Chemical Changes during Cleavage:

Significant chemical changes go on in the fertilized egg during cleavage.

They are:

Increase of nuclear material:

During cleavage a steady increase in nuclear material (predominantly DNA) is observed. Cytoplasm of the egg is the source of such nuclear material. Cytoplasmic DNA contained in mitochondria and yolk platelets are available.

RNA synthesis:

During cleavage messenger RNA (mRNA) and transfer RNA (tRNA) are synthesised during cleavage, especially in late stages.

Synthesis of proteins:

Throughout the period of cleavage there is steady and spectacular increase in protein synthesis.

---

7. Cleavage in Different Chordates:

The pattern of cleavage differs in different animals. The following account will give an idea of the process of cleavage in different chordates.

a. Amphioxus:

The cleavage in Amphioxus is typically holoblastic (Fig. 5.10). The first cleavage is meridional. The second cleavage is also meridional but at right angle to the first one. Four equal blastomeres are produced. The third cleavage is latitudinal and occurs slightly above the equatorial plane resulting in the production of eight blastomeres—four are smaller called the micromeres and four are larger known as the macromeres.

The micromeres are situated towards the animal pole and the macromeres towards the vegetal pole. The fourth cleavage is meridional which involves all the eight cells resulting in the formation of eight micromeres and eight macromeres. The fifth cleavage planes are latitudinal.

Each micromere is divided into an upper and lower micromere and each macromere likewise divides to form an upper and lower macromere. The fifth cleavage planes produce thirty-two blastomeres. The sixth cleavage planes are nearly meridional involving all the thirty-two cells resulting in sixty-four cells.

At the 64-cell stage a conspicuous space is produced at the centre and this space becomes filled with a fluid. When the eighth cleavage planes take place, the blastula becomes pear- shaped and the blastocoel becomes large.

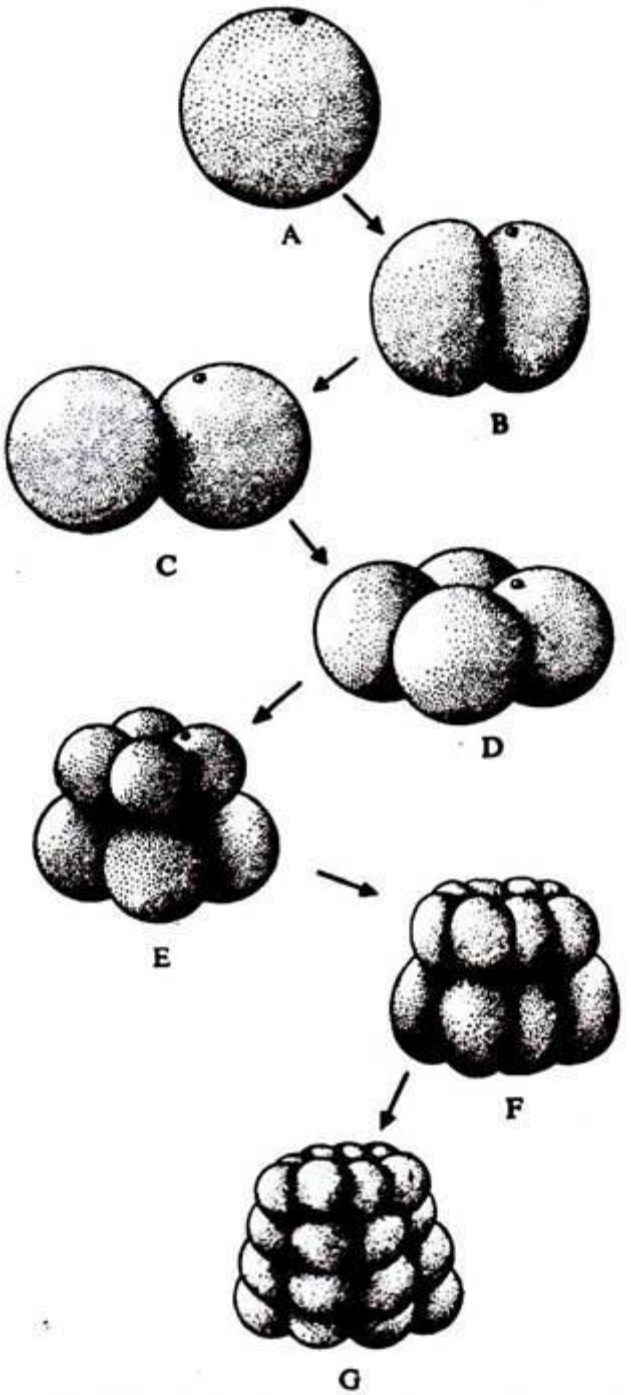


Fig. 5.10. Early cleavage pattern in the egg of Amphioxus. A. Fertilized egg. B–C. First cleavage. D. Second cleavage. E. Third cleavage. F. Fourth cleavage. G. Morula stage.

b. Frog:

The egg of frog is telolecithal with a considerable amount of yolk localized towards the vegetal pole. The cleavage is holoblastic in nature, but differs considerably from that of Amphioxus because of larger quantity of yolk.

The first cleavage plane is meridional which occurs at about 3-3½ hours after fertilization. But the time depends largely on extrinsic factors. The first cleavage starts at the animal pole and gradually travels towards the vegetal pole. Thus the egg is bisected along the poles. Two blastomeres of equal size are produced. The second cleavage is almost meridional but oriented at right angles to the first cleavage plane (Fig. 5.11).

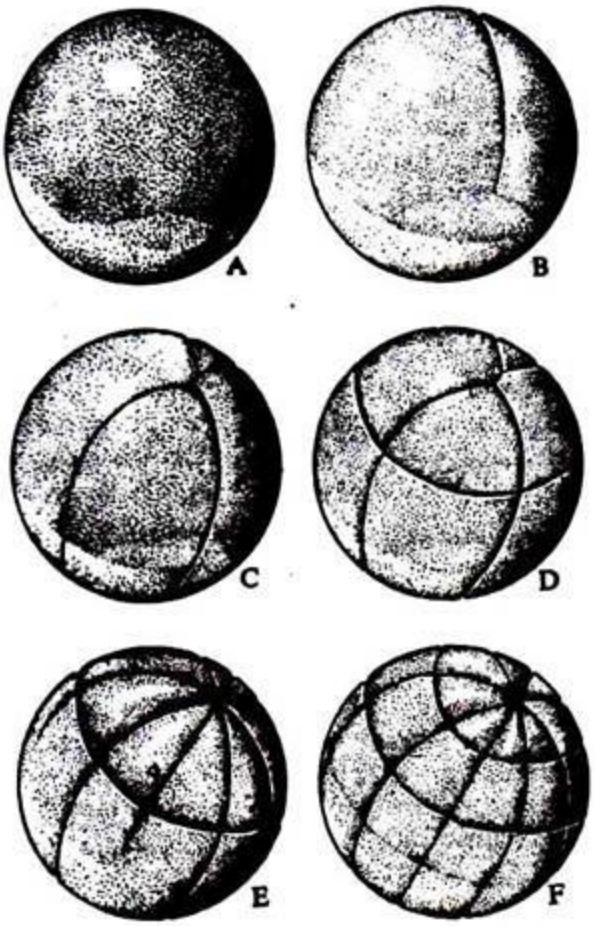


Fig. 5.11. Semidiagrammatic representation of the cleavage pattern in the egg of frog. A. Fertilized egg. B. First cleavage. C. Second cleavage. D. Third cleavage. E. Fourth cleavage. F. Morula stage.

The four blastomeres thus produced are not qualitatively identical, because the grey crescent material is present in two of the four blastomeres. Each blastomere contains dark pigment at the animal pole and yellowish yolk towards the vegetal pole. The third cleavage is latitudinal and occurs at right angles to previous cleavage planes but passes slightly above the equator.

The furrow produces eight unequal blastomeres, four micromeres in the animal hemisphere and four macromeres in the vegetal part. The fourth cleavage planes are meridional which involve the micromeres first and pass on slowly towards the yolk-laden macromeres of the vegetal pole.

In Amphioxus, the cleavages occur in a synchronous fashion, while in frog considerable degree of irregularities (asynchronism) appear in later stages. But it is certain that the micromeres always continue to divide at a faster rate than do the macromeres.

At the eight-celled stage, a small space makes its appearance between the four micromeres. As development goes on, this space becomes conspicuous and forms the blastocoel. The floor of the blastocoel is formed of macromeres. The blastocoel (or segmentation cavity) is eccentrically located and becomes displaced towards the animal pole as development proceeds.

c. Chick:

Typical meroblastic cleavage occurs in chick, where the segmentation activity is restricted only at the blastodisc or germinal disc (Fig. 5.12). Thus the cleavage is incomplete.

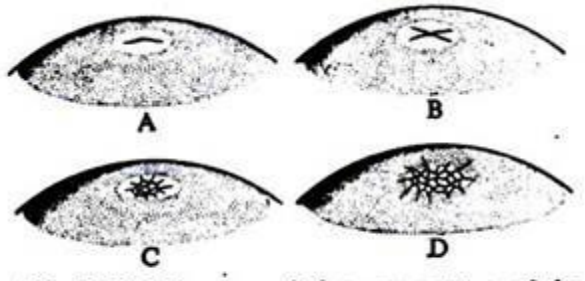


Fig. 5.12. Side view of cleavage pattern of the egg of chick. A. First cleavage. B. Second cleavage. C–D. Subsequent cleavages.

The first cleavage starts as a meridional furrow near the centre of the blastodisc at about 4½ hours after fertilization when the egg reaches the isthmus of oviduct. This furrow cuts across the blastodisc and passes towards the vegetal pole but does not reach the pole. The second cleavage is also meridional, but approximately at right angles to the first one. The third cleavage is vertical.

The fourth cleavage is also vertical but the division is not synchronous. As a consequence eight central cells encircled by twelve marginal cells are produced. From this point onward the cleavage becomes irregular and a disc containing smaller cells appears.

This disc remains firmly connected with the underlying yolk. Soon a cleft appears which separates the disc in the middle from the underlying yolk. The new cavity in between is known as sub-germinal space (Fig. 5.13).

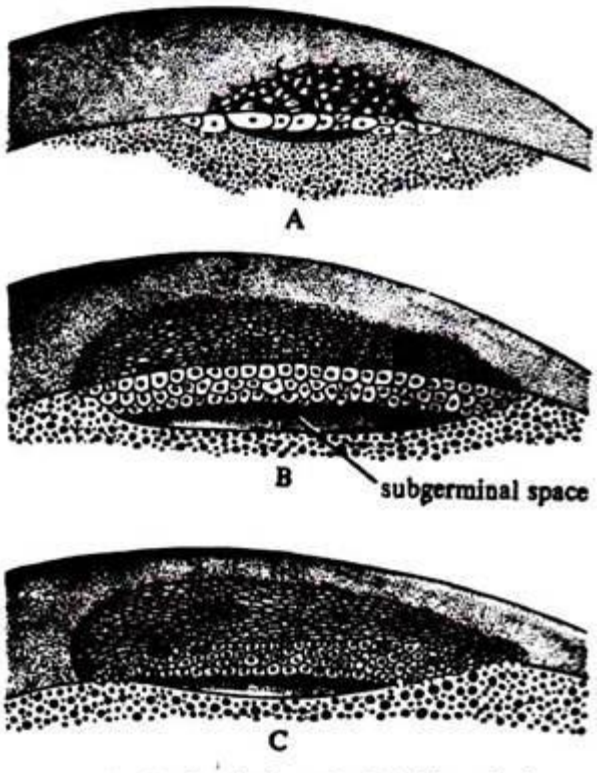


Fig. 5.13. Sectional view of chick blastula showing the formation (A–C) of subgerminal space (after Huettner).

Thus at the end of segmentation, the disc contains many-layered small cells which are connected with the yolk only at the periphery. This disc is then termed as blastoderm, the cells of which still continue to divide.

The peripheral part which lies in contact with yolk possesses granular cells called area apaca and the inner layer having clear portion is called area pellucida. At one end of area opaca, aggregation of cells takes place. This denotes the formation of future posterior side.

d. Rabbit:

The egg of rabbit is small and does not contain any yolk (i.e. alecithal type of egg). The cleavage is holoblastic and nearly equal. Irregularities and a synchronism become the rule in the cleavage of rabbit like all other eutherian mammals.

The first cleavage is vertical resulting in the formation of two unequal blastomeres. The second cleavage is also vertical but runs at right angle to the first. The third cleavage is horizontal but slightly above the equator.

Subsequent divisions are rapid and irregular. The blastomeres thus produced become clustered together to form a solid cellular ball called morula. Two types of cells (small and large) are recognised in the morula.

The large cells lie at the centre. Soon a cavity appears inside the cell mass on one side. The cavity gradually increases which shifts the central cell mass to one side. The stage is called blastocyst stage. The inner cell mass in the centre is attached with the outer cell layer (trophoblast) of the blastocyst.

The cavity is called the blastocoel or sub-germinal cavity (Fig. 5.13A) which is filled with a fluid. The inner cell mass remains attached at the embryonic knob towards the animal pole. From this embryonic knob, the embryo arises. The trophoblast which encloses the blastocoel and the embryonic knob participates in the formation of placenta. The trophoblastic cells overlying the embryonic knob is called cells of Rauber.

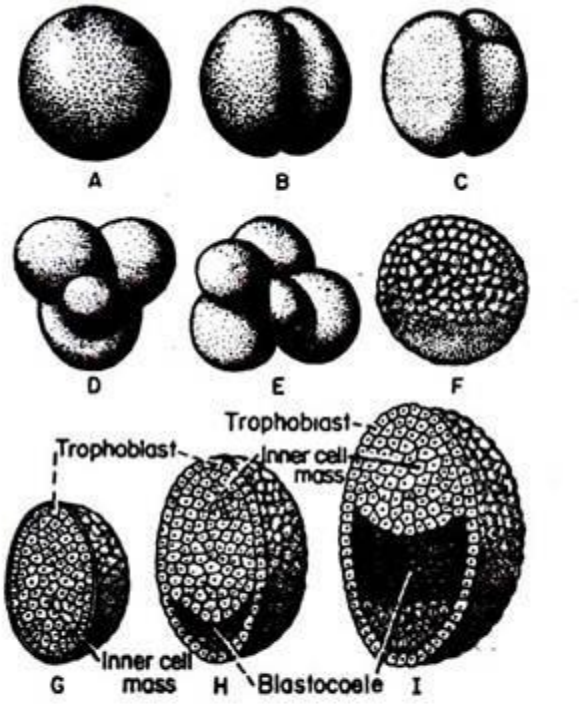


Fig. 5.13A. Showing the process of cleavage in Rabbit.
A=Zygote, B–E=Cleavage stages, F=Morula, G=Section of morula, H & I=Differentiation of inner cell mass and Trophoblast.

8. Importance of Cleavage in Embryonic Pattern:

The cleavage phase of development and blastulation are extremely significant, because the blastoderm is morphologically elaborated in such a way that the important presumptive organ forming areas of the future embryo are segregated into definite districts of the blastoderm.

Such orientation of the organ forming areas in the blastoderm permits an ordered movement of these areas during gastrulation to take up their fateful position. So the period of cleavage and blastulation is regarded as the phase of preparation for future differentiation.

The cells which are produced at the end of segmentation resemble the zygote—but do they possess the same potentiality as the zygote itself. Driesch (1891), in order to get an answer, separated the two blastomeres at the two-celled stage and found that both the blastomeres developed into complete embryos.

His conclusion was that each blastomere has the full potentiality to be an entire embryo. But in 1900, Roux showed that if one of the blastomeres of the two-celled stage is killed, the remaining one produces ‘half embryo’.

He claimed that each cleavage results into the segregation of specialization in the blastomeres and this is irreversible. This experiment demonstrates that an organising or controlling centre is elaborated to control the development process.

The experiment of Spemann and others have shown that it is the grey crescent region which plays the vital role in the process of determination and the blastomeres which are formed due to segmentation are neither completely regulative nor irreversibly determined.

Fig. 5.14 shows the importance of grey crescent in the development of amphibian embryo. It has been experimentally established that the grey crescent in the amphibian blastula transforms into the dorsal lip of the blastopore which acts as an instigator and controller of the gastrulation process.

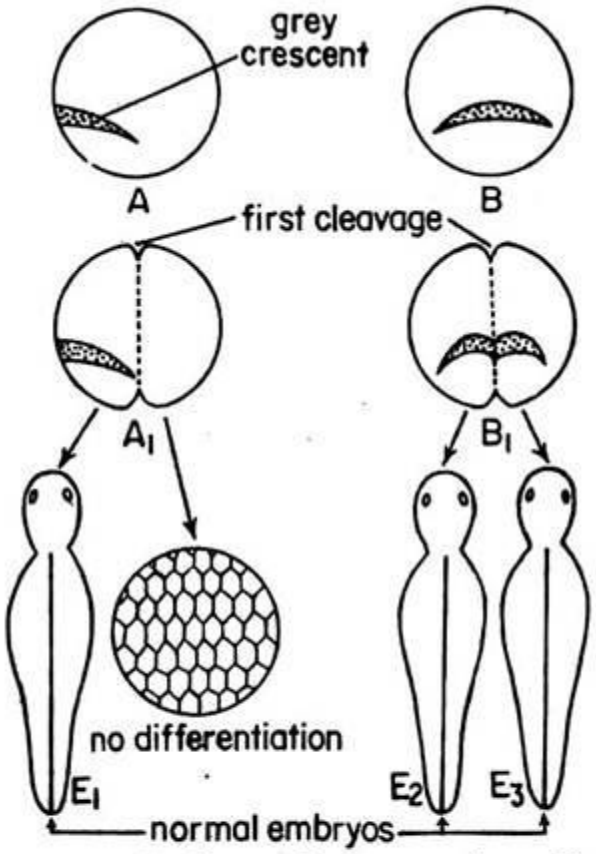


Fig. 5.14. Significance of grey cresent in amphibian development. A. Side view. B. Top view. A_1. Separation of two blastomeres, one with grey crescent and the other without grey crescent. The blastomere lacking the grey crescent fails to differentiate. B_1. Both the blastomeres contain grey crescent and develop into normal embryos. E_1–E_3 Normal embryos.

TABLE EMBRYOLOGY–2

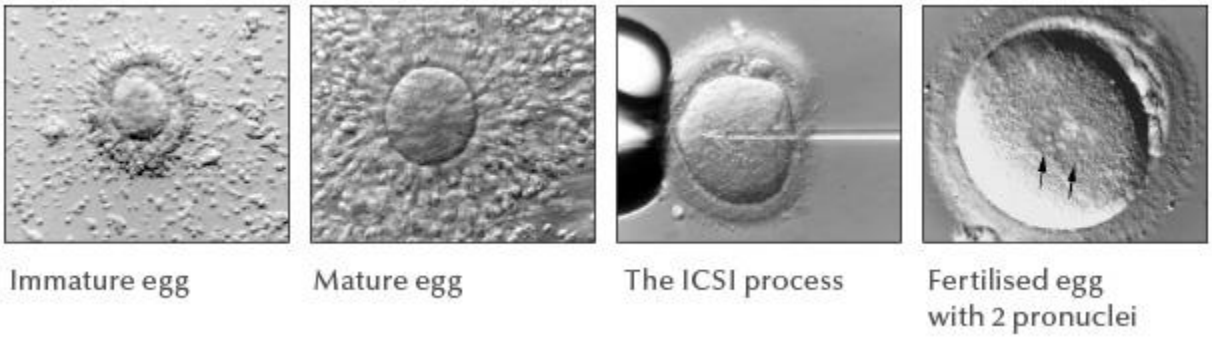
Comparative account of cleavage in Amphioxus, Frog and Chick

Points	Amphioxus	Frog	Chick
1. Type of cleavage	1. Holoblastic	1. Holoblastic	1. Meroblastic
2. First cleavage plane	2. Meridional (Equal)	2. Meridional (Equal)	2. Meridional (Equal)
3. Second cleavage plane	3. Meridional, but at right angle to first one (Equal)	3. Same as Amphioxus	3. Same as Amphioxus
4. Third cleavage plane	4. Latitudinal, slightly above the equator of egg (unequal)	4. Same as Amphioxus	4. Vertical
5. Fourth cleavage plane	5. Meridional (Equal)	5. Meridional (Equal)	5. Vertical
6. Fifth cleavage plane	6. Latitudinal (unequal)	6. Irregular	6. Irregular
7. Sixth cleavage plane	7. Nearly meridional	7. Irregular	7. Irregular
8. Regularity in cleavage	8. Synchronism prevails upto sixth cleavage.	8. Asynchronism starts from fifth cleavage.	8. Asynchronism becomes the rule from the fifth cleavage
9. Blastula	9. Becomes pear-shaped with a large and spacious blastocoelic cavity. The wall is composed of one layer of cells.	9. Rounded and the blastocoelic cavity is located towards the animal pole. The epiblast has micromeres while the hypoblast has macromeres.	9. Cleavage is restricted to a round blastodisc, which is demarcated from the underlying yolk by subgerminal space. The blastodisc is divided into two zones—(*i*) area pellucida and (*ii*) area opaca.

In vitro fertilization (IVF)

At the time of egg collection, our scientists are looking for mature eggs with smooth borders and a fluffy corona of cumulus cells encircling them. Immature eggs, or those that have gone past optimum maturity (postmature), have irregular borders and poorer quality cumulus.

In conventional in vitro fertilisation (IVF), about 50,000 to 100,000 washed sperm are left in a small dish with the mature eggs. The sperm spend the next few hours attempting to penetrate the corona of cumulus cells. Hopefully one sperm will be successful and fertilise the egg.



Immature egg Mature egg The ICSI process Fertilised egg with 2 pronuclei

Intracytoplasmic sperm injection (ICSI)

A special technique called intracytoplasmic sperm injection (ICSI) can be used to fertilise eggs when there is thought to be a limited opportunity of fertilisation occurring with conventional IVF. This might be either because of problems with low sperm numbers or low sperm motility, or because of other barriers to the fertilisation process such as sperm antibodies or previous failure to fertilise through IVF.

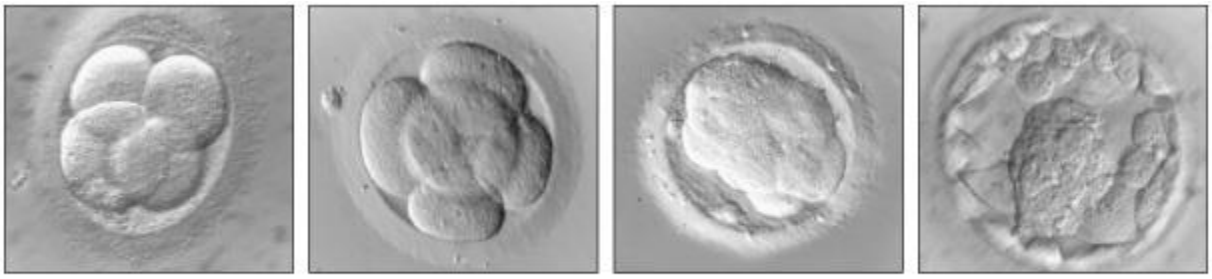
During ICSI, a single sperm is injected into each egg. The sperm is selected mainly on the basis of its normal shape and size.

Blastocyst culture

Once fertilisation has occurred the embryo will divide and rapidly increase in cell numbers over the next few days.

By Day 4, the cells have divided rapidly but the embryo has not yet increased in size. It is now 'compacting' (you can't distinguish the cells) and is called amorula.

If the embryo survives to Day 5 – the blastocyst stage – it will contain between 75 and 100 cells. It is a 3-dimensional ball of outer cells (the trophectoderm) surrounding a fluid-filled cyst in which an inner group of cells (the inner cell mass) can be seen.



Day 2 3-4 cells **Day 5** blastocyst

The trophectoderm will go on to form the placenta, membranes and umbilical cord, while the inner cell mass will become the baby. It is not possible to tell the difference between a 'good' embryo and a 'bad' embryo just by looking at them. Embryos at the best of times are busy transforming and repairing themselves, so can develop fragmentation (when small bits of cells are pinched off during division) or vacuoles (which are small spaces within the substance of the cells). The significance of these changes is not known and many fragmented and vacuolated embryos can go on to form perfectly healthy pregnancies.

While many embryos can survive 2 or 3 days to reach the 4-8 cell stage, only the strongest will have the ability to keep developing into a blastocyst and then a baby. One way of identifying the better embryos, therefore, is to let them grow a little longer in the laboratory and to transfer them

at the blastocyst stage. This is a good way of determining which embryos have the most developmental potential and maximising the potential of a successful pregnancy.

Our great success with blastocyst culture and implantation means that blastocyst transfer is a standard part of our service at Genea Hollywood Fertility.

Energy

An embryo's energy supply comes from tiny structures inside its cells called mitochondria. Until it has implanted and formed a placenta, the embryo relies on the energy produced by the mitochondria it has inherited from its egg. Because females are born with all the eggs for their lifetime, the egg mitochondria are essentially the same age as the woman herself.

Chromosomes

Embryos must also have the right genetic makeup to develop normally. In humans, genes are contained in 23 pairs of chromosomes. An incorrect number of chromosomes leads to failure of an embryo to implant or to progress to a healthy baby. Pregnancy is a great filter of abnormal embryos. When chromosome analysis is performed on cells from Day 3 embryos, studies have shown that only one third will have the normal number. If an embryo progresses to Day 5 and becomes a blastocyst, it has a two-thirds chance of being chromosomally normal. 90% of chromosomally abnormal pregnancies will miscarry in the first trimester (first 12 weeks of pregnancy). 93% of chromosomally normal pregnancies will continue to term.

Ovarian stimulation

Ovulation induction (OI) with controlled ovarian stimulation may be recommended for women who have normal tubes, and whose partners have a normal semen analysis, but who rarely or never ovulate. For women who do ovulate regularly, stimulation can also be used to maximise the potential of pregnancy by increasing the number of follicles that develop fully and, therefore, increasing the number of eggs that are ovulated during a cycle.

Two types of hormones may be used to stimulate ovulation:

- Tablets of Letrozole®
- Injections of follicle stimulating hormone (FSH) – Gonal-F®, Puregon® and Menopur®

Letrozole

Letrozole can potentially be a first choice treatment for stimulating ovulation for women under 30 years of age because of it's low cost and ease of use. Normally Letrozole is a medication that is used for the treatment of breast cancer. It is an aromatase inhibitor and works by lowering the production of oestrogen.

When the enzyme aromatase is inhibited by Letrozole, oestrogen levels are suppressed. This results in the brain and pituitary gland increasing the output of FSH (follicle stimulating hormone).

In women with PCOS or anovulation (a problem with ovulation) the increase in FSH hormone encourages development of a mature follicle in the ovary and ovulation of an egg. This process is called ovulation induction.

Side effects can include hot flushes and headaches however the treatment duration for Letrozole is only five days so these should be short-lived. Other possible side effects include fatigue, nausea, constipation, diarrhea, joint pain.

As with any medication that stimulates the ovaries, there is an increased risk of multiple pregnancy. Studies have indicated that approximately 4% of the pregnancies conceived with Letrozole are twins.

Follicle stimulating hormone (FSH)

FSH is the hormone necessary for the development of the multiple follicles required for in vitro fertilisation (IVF).

FSH may also be used in smaller doses for ovulation induction or ovarian hyperstimulation in IVF. The FSH is made in the laboratory and is identical to human FSH. Because it is a protein that if taken orally would be digested in the stomach, FSH is given by injection under the skin, with a fine needle. There are three brands of FSH available in Australia – Gonal-F®, Puregon® and Menopur®. All are self-administered with pen-like devices (similar to those used for insulin by diabetics).

Using FSH to induce ovulation for getting pregnant naturally, as opposed to through IVF, can be tricky because of the risk of stimulating too many follicles and having a multiple pregnancy. This is why the body's response is closely monitored with blood tests and ultrasounds.

When the lead follicle or follicles are the right size on ultrasound, ovulation is triggered with an injection of human chorionic gonadotrophin (hCG), which mimics the luteinising hormone (LH) surge.

Even with the most careful monitoring, more follicles can reach maturity than desired. Intercourse should be avoided because of the high risk of twins, triplets or an even higher-order multiple pregnancy. If this looks too likely, either the ovulation cycle that has been induced will need to be cancelled or a suggestion might be made to carry out an IVF procedure instead, in a separate treatment cycle.

Possible Questions

2 Marks

Define Embryology

What is zygote?

What is gametogenesis?

8 Marks

Explain Collection and preservation of embryos

Discuss about culturing of embryos

Describe about types of cleavage pattern

Describe every steps in *In vitro* fertilization

Describe stem cell research and its application

UNIT -V

Transgenics: Transgenic animals; production and application; transgenic animals as models for human diseases; transgenic animals in live- stock improvement; expression of the bovine growth hormone; transgenics in industry. Ethical issues in animal biotechnology.

Why transgenic animals are used?

- Selective breeding is performed since centuries.
- Breeding is time consuming.
- Crossing in properties is time consuming.
- Only a limited number of properties available
- Introduction of a desired property (hypothesis driven; without hypothesis)
- Fast generation of animal lines carrying the desired property
- Animal model for human diseases
- Animal system to produce biomolecules (Pharming)
- Xeno-Transplantates

Transgenesis in Mice – Methodology

- A genetically modified mouse (Mus musculus) is a mouse that has had its genome altered through the use of genetic engineering techniques.
- Genetically modified mice are commonly used for research or as animal models of human diseases, and are also used for research on genes.
- There are two basic technical approaches to produce genetically modified mice. The first involves pronuclear injection into a single cell of the mouse embryo, where it will randomly integrate into the mouse genome.
- This method creates a transgenic mouse and is used to insert new genetic information into the mouse genome or to over-express endogenous genes.

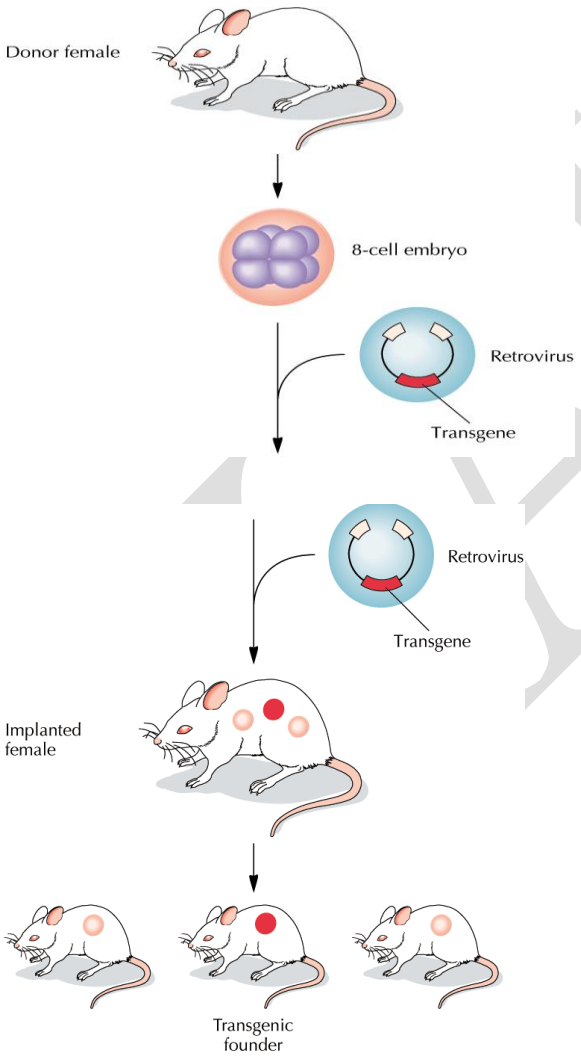
- The second approach, pioneered by Oliver Smithies and Mario Capecchi, involves modifying embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene. Embryonic stem cells that recombine with the genomic DNA are selected for and they are then injected into the mice blastocysts.
- This method is used to manipulate a single gene, in most cases "knocking out" the target gene, although more subtle genetic manipulation can occur (e.g. only changing single nucleotides)

Transgenic Mouse – Uses and applications

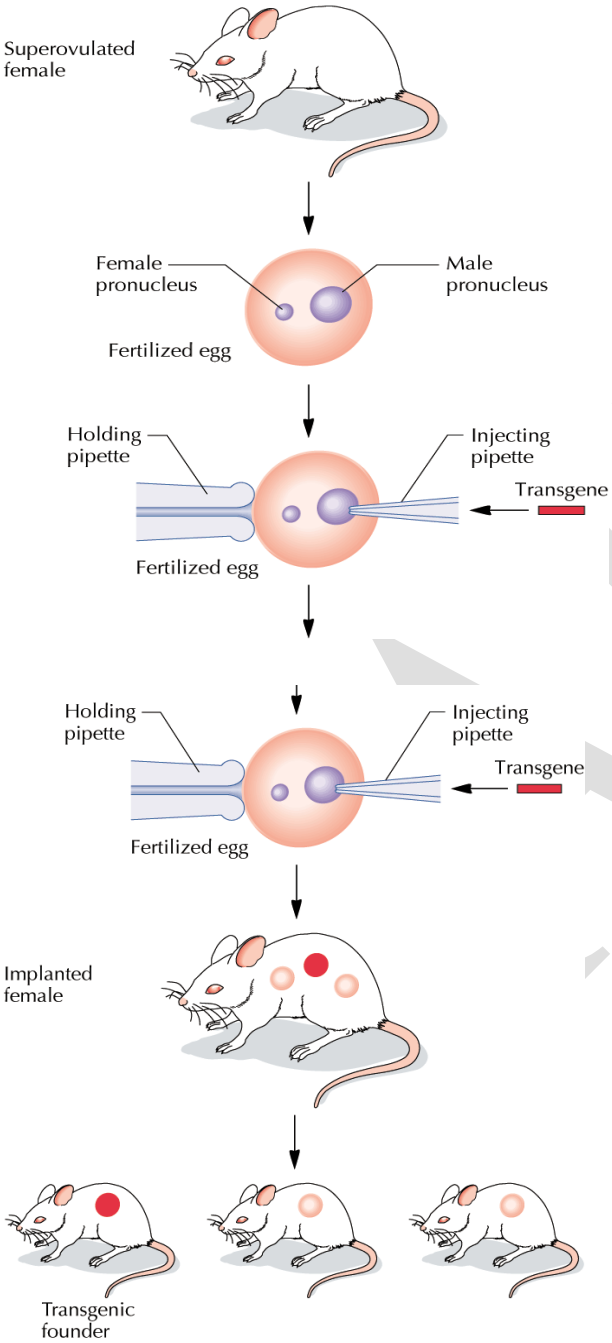
- Genetically modified mice are used extensively in research as models of human disease. Mice are a useful model for genetic manipulation and research, as their tissues and organs are similar to that of a human and they carry virtually all the same genes that operate in humans.
- They also have advantages over other mammals, in regards to research, in that they are available in hundreds of genetically homogeneous strains. Also, due to their size, they can be kept and housed in large numbers, reducing the cost of research and experiments.
- The most common type is the knockout mouse, where the activity of a single (or in some cases multiple) genes are removed. They have been used to study and model obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease.
- Transgenic mice generated to carry cloned oncogenes and knockout mice lacking tumor suppressing genes have provided good models for human cancer. Hundreds of these oncomice have been developed covering a wide range of cancers affecting most organs of the body and they are being refined to become more representative of human cancer. The disease symptoms and potential drugs or treatments can be tested against these mouse models.

- A mouse has been genetically engineered to have increased muscle growth and strength by overexpressing the insulin-like growth factor I (IGF-I) in differentiated muscle fibers.
- Another mouse has had a gene altered that is involved in glucose metabolism and runs faster, lives longer, is more sexually active and eats more without getting fat than the average mouse (see Metabolic supermice).

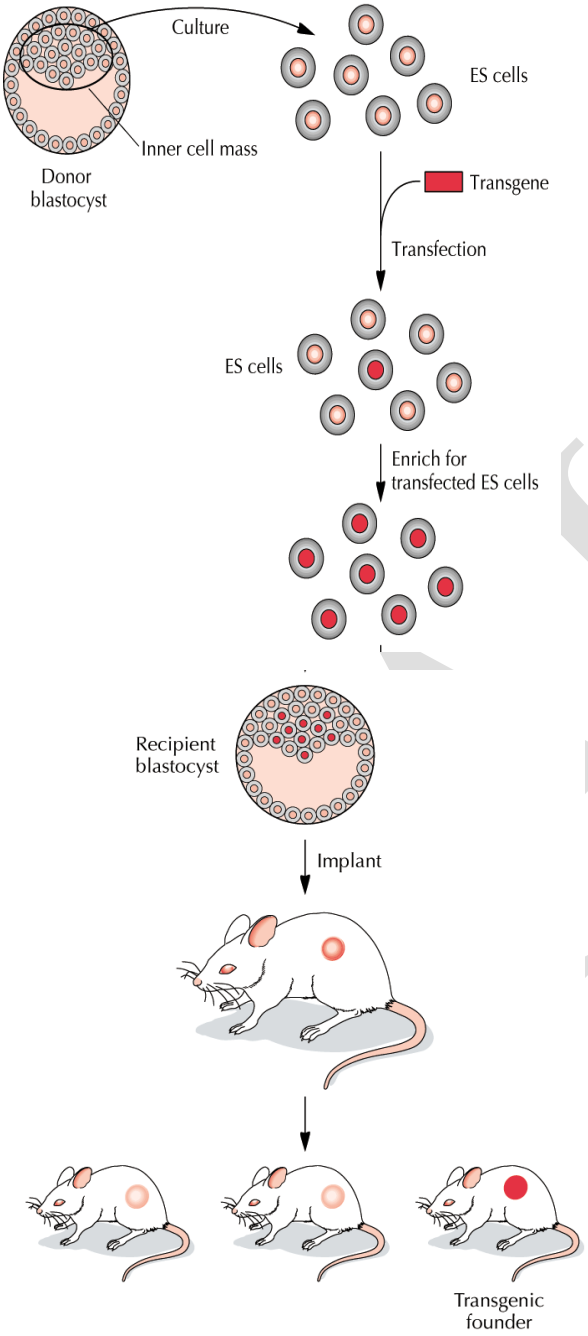
Retroviral Vector



Microinjection



Stem cell Method



Transgenic Cow

Transgenic cows are genetically modified (GM) cows. They have an extra gene or genes inserted into their DNA. The extra gene may come from the same species or from a different species.

The extra gene (transgene) is present in every cell in the transgenic cow. However, it's only expressed in mammary tissue. This means that the transgene's protein will only be found in the cow's milk and can only be extracted from there.

Techniques used to make transgenic cows

Step 1. Designing the gene construct

The first step is to design a gene construct. The gene construct is a unit of DNA that includes:

- an antibiotic resistance gene – to select cells that have taken up the gene construct
- a tissue-specific promoter sequence – to signal the start of expression of the protein in cells of the appropriate tissue, for example, in mammary cells in lactating cows
- the desired gene – for example, bovine casein or human myelin basic protein
- a stop sequence – to define the end of the information for making the protein**.**

Step 2. Sourcing the transgene

- In the past, the gene would have been extracted from the source organism's DNA. Now, however, if the desired gene sequence is known, it can simply be synthesised in a lab.
- There are companies that make genes to order within a couple of weeks.

Step 3. Making the gene construct

- A gene construct contains all the information needed for transfection into a bovine cell and expression of the desired gene in a cow. This includes an antibiotic resistance marker, a tissue-specific promoter, the transgene/gene of interest and a stop sequence.

- The gene is usually supplied in a vector. A vector is a small piece of DNA, often a plasmid, into which a foreign piece of DNA can be inserted. When the gene of interest is in a vector, it can be sent from one lab to another, it can be stored, it can be manipulated or it can be used to transform bacteria to produce more copies of the gene of interest.
- Vectors have multiple restriction enzymes sites (also called multiple cloning sites) so the gene can be inserted into the vector and then cut out from the vector using restriction enzymes. This article has more information on restriction enzymes.
- After the gene is cut from the vector, it is then pasted into the multiple cloning site of the gene construct using a method known as ligation. This article has more information on DNA ligation.

Step 4. Transfecting bovine cells

- The gene construct is incorporated into the genome of a bovine (cow) cell using a technique called transfection. During transfection, holes are made in the cell membrane that allow the DNA to enter.
- The holes can be made by applying an electrical pulse or by adding chemicals to the cells. Once inside the cell, the gene construct may enter the nucleus and incorporate into the cell's genome.

Step 5. Selecting for transgene positive cells

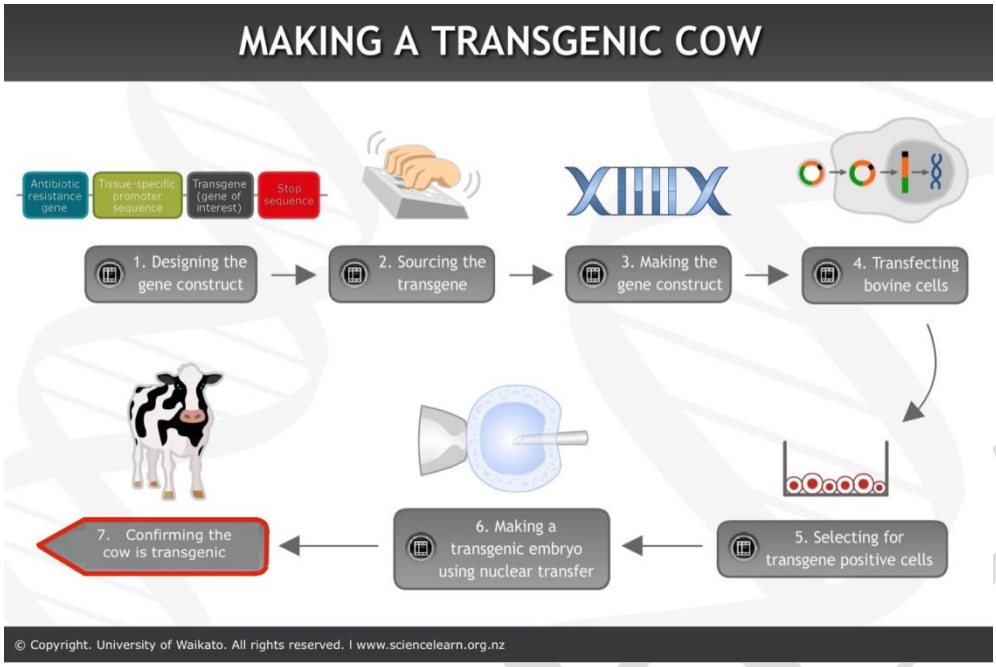
- After transfection, an antibiotic is added to select the bovine cells that have incorporated the gene construct. Transgenic bovine cells will survive treatment with an antibiotic, because they contain an antibiotic resistance gene making them resistant to the antibiotic.
- Cells without the gene construct will have no resistance to the antibiotic and will die. In addition to antibiotic selection, polymerase chain reaction (PCR) is used to check that the bovine cell contains the transgene.

Step 6. Making a transgenic embryo using nuclear transfer

- Nuclear transfer is used to create a whole animal from a single transgenic bovine cell.
- The transgenic bovine cell is fused with a bovine oocyte that has had its chromosomes removed (called an enucleated oocyte). An electrical pulse is applied to help fuse the cells. Once fused with the oocyte, the transgenic cell's chromosomes are reprogrammed to direct development into an embryo.
- After 7 days, the transgenic embryo will have about 150 cells and can be transferred into a recipient cow for further development to term.

Step 7. Confirming the cow is transgenic

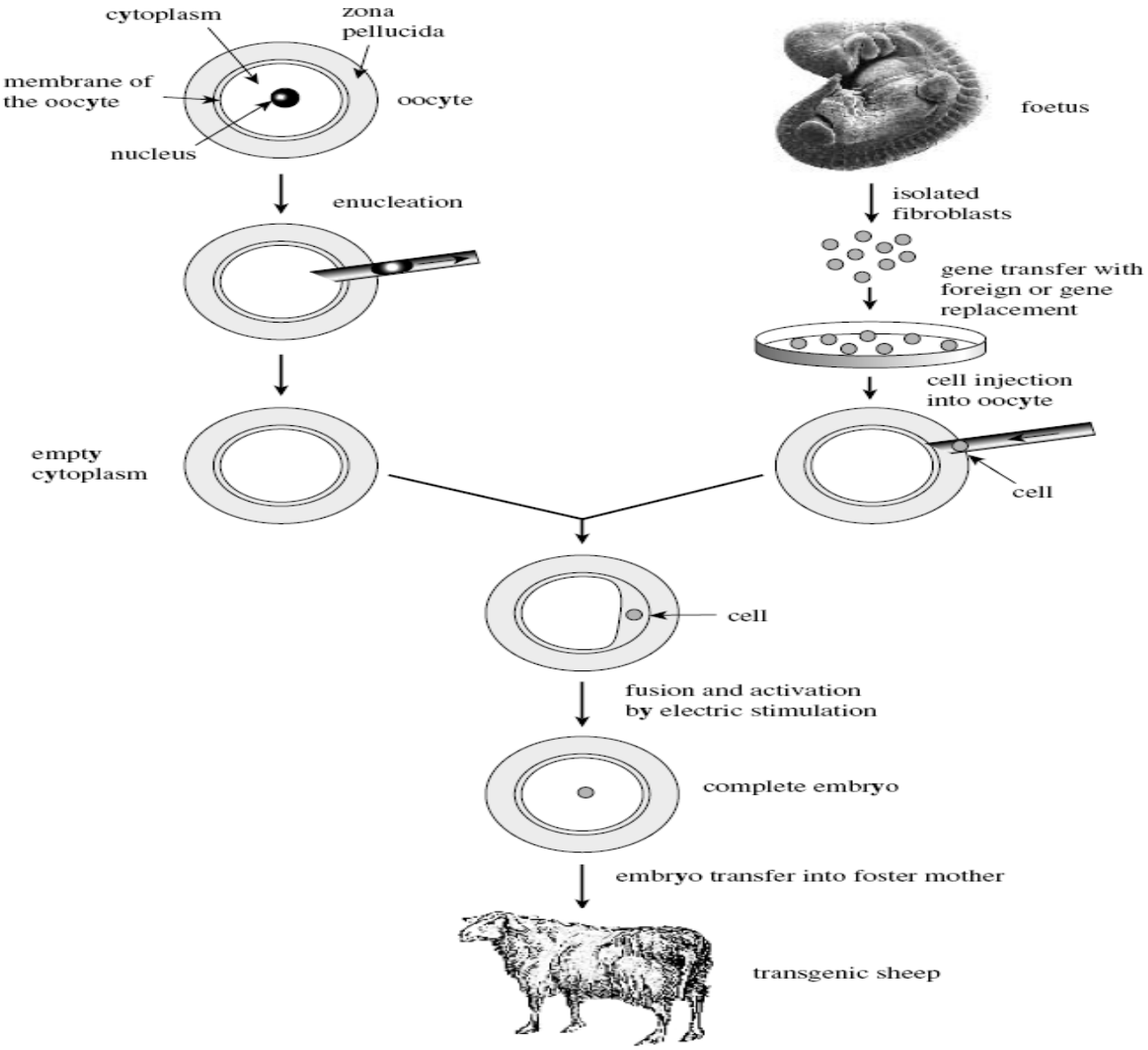
- If the embryo develops to full term, after 9 months, the cow will give birth to a calf. To confirm that the calf is transgenic, scientists can check using:
- PCR to determine the presence or absence of the transgene
- quantitative PCR (q-PCR) to determine the number of copies of the transgene
- fluorescent in situ hybridization (FISH) to visualize where the transgene is in the chromosome and whether the transgene has integrated into more than one chromosome.
- When the calf is lactating (either after being induced to lactate or after having its own progeny), its milk is checked to determine if the transgenic protein is being expressed.



Transgenic sheep, goat

The birth of Dolly was soon followed by that of a cloned transgenic sheep named Polly (Schnieke et al., 1997). The advantages of this method of adding genes are multiple. About two to five times fewer sheep are needed to generate transgenic sheep than by microinjection. The integrated gene may be examined in cells before nuclear transfer. Cells in which the foreign gene is rearranged or has too many copies may be discarded. The sex and, more generally, the genotype of the nuclear donors may be chosen.

The founder animals are never mosaic for the transgene. Several animals having the same genotype, including the same transgene, can be generated simultaneously. Although cloning is a laborious technique, it offers some flexibility to the experimenters. The nuclear donor cells can be kept frozen and used at the most appropriate moment to generate cloned transgenic animals.



Transgenic Sheep and Goats

Until recently, the transgenes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a transgene into a specific

gene locus was reported. The gene was the human gene for alpha1-antitrypsin, and two of the animals expressed large quantities of the human protein in their milk.

This is how it was done

Sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA:

1. 2 regions homologous to the sheep *COL1A1* gene. This gene encodes Type 1 collagen. (Its absence in humans causes the inherited disease osteogenesis imperfecta)
   This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.
2. A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector. The human gene encoding alpha1-antitrypsin.
   Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease **Alpha1-Antitrypsin Deficiency** (**A1AD** or **Alpha1**). The main symptoms are damage to the lungs (and sometimes to the liver).
3. Promoter sites from the **beta-lactoglobulin** gene. These promote hormone-driven gene expression in milk-producing cells.
4. Binding sites for ribosomes for efficient translation of the mRNAs.

Successfully-transformed cells were then

- fused with enucleated sheep eggs and
- implanted in the uterus of a ewe (female sheep).
- Several embryos survived until their birth, and two young lambs have now lived over a year.
- When treated with hormones, these two lambs secreted milk containing large amounts of alpha1-antitrypsin (650 µg/ml; 50 times higher than previous results using random insertion of the transgene).

On June 18, 2003, the company doing this work abandoned it because of the great expense of building a facility for purifying the protein from sheep's milk. Purification is important because

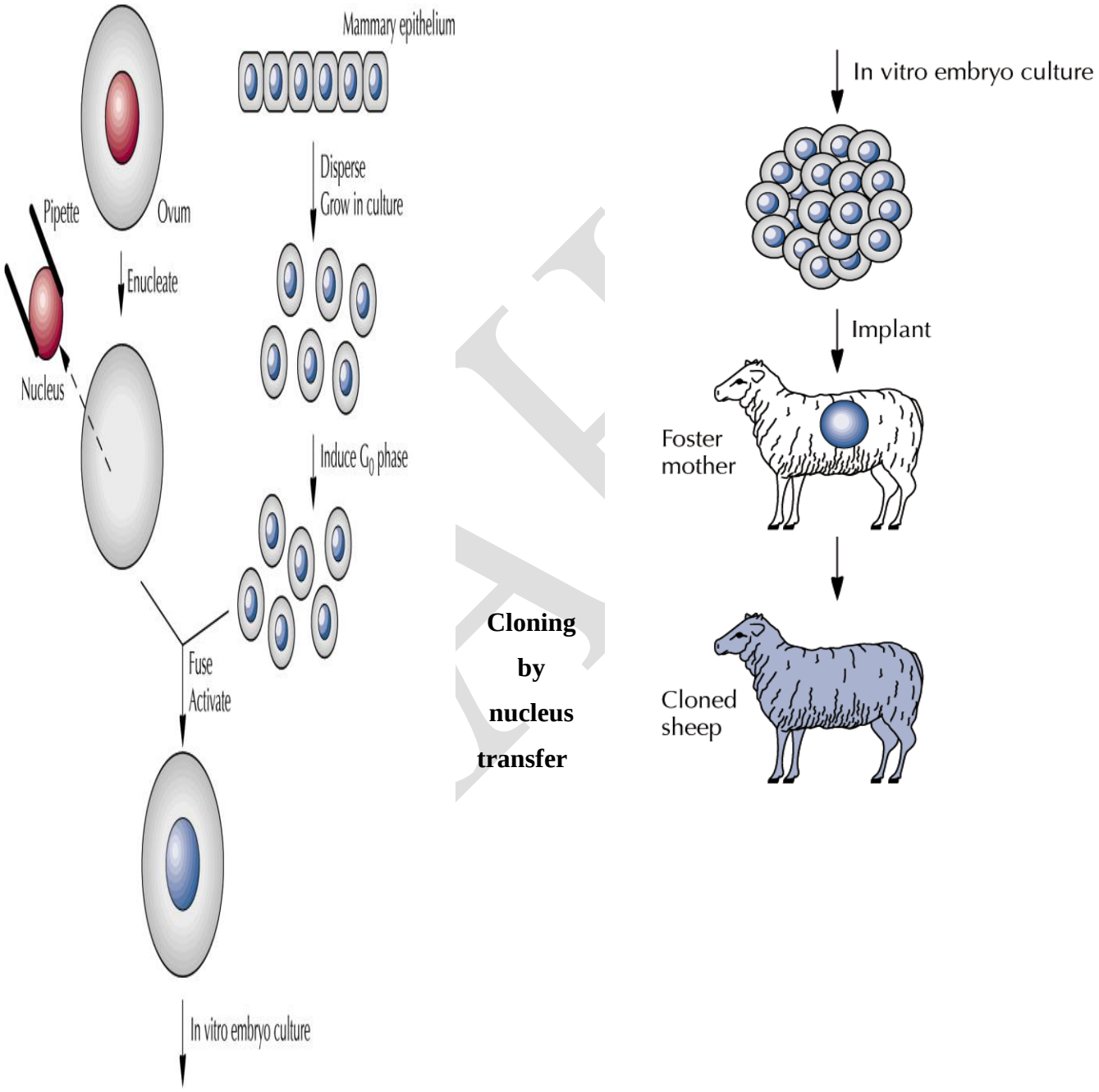
even when 99.9% pure, human patients can develop antibodies against the tiny amounts of sheep proteins that remain.

However, another company, GTC Biotherapeutics, has persevered and in June of 2006 won preliminary approval to market a human protein, antithrombin, in Europe. Their protein — the first made in a transgenic animal to receive regulatory approval for human therapy — was secreted in the milk of transgenic goats.

Transgenic Pigs

Transgenic pigs have also been produced by fertilizing normal eggs with sperm cells that have incorporated foreign DNA. This procedure, called sperm-mediated gene transfer (SMGT) may someday be able to produce transgenic pigs that can serve as a source of transplanted organs for humans.

Gene replacement was achieved in sheep (McCreath et al., 2000), mice
(Rideout et al., 2000) and pigs (Lai et al., 2002; Butler, 2002). This method is very laborious and still poorly controlled. A recent study showed that homologous recombination of two genes could be obtained in sheep cells but that this was followed by the death of the newborn animals obtained by cloning (Denning et al., 2001). This failure may be attributed to the culture of the cells, which is required to select those in
which the homologous recombination has occurred. The culture conditions modify the physiology of the cells, which, for unknown reasons, become less capable of generating living cloned animals. A better understanding of these phenomena is necessary before gene replacement in large animals can be considered as a truly viable method.



Cloning by nucleus transfer

Transgenic fish

Genetically modified fish (GM fish) are organisms from the taxonomic clade which includes the classes Agnatha (jawless fish), Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish) whose genetic material (DNA) has been altered using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the fish which does not occur naturally in the species, i.e. transgenesis.

The first transgenic fish were produced in China in 1985. As of 2013, approximately 50 species of fish have been subject to genetic modification. This has resulted in more than 400 fish/trait combinations. Most of the modifications have been conducted on food species, such as Atlantic salmon (*Salmo salar*), tilapia (genus) and common carp (*Cyprinus carpio*).

Generally, genetic modification entails manipulation of DNA. The process is known as cisgenesis when a gene is transferred between organisms that could be conventionally bred, or transgenesis when a gene from one species is added to a different species. Gene transfer into the genome of the desired organism, as for fish in this case, requires a vector like a lentivirus or mechanical/physical insertion of the altered genes into the nucleus of the host by means of a micro syringe or a gene gun

Transgenic fish are used in research covering five broad areas-

- Enhancing the traits of commercially available fish
- Their use as bioreactors for the development of bio-medically important proteins
- Their use as indicators of aquatic pollutants
- Developing new non-mammalian animal models
- Functional genomics studies

Most GM fish are used in basic research in genetics and development. Two species of fish, zebrafish and medaka, are most commonly modified because they have optically clear chorions (shells), develop rapidly, the 1-cell embryo is easy to see and micro-inject with transgenic DNA, and zebrafish have the capability of regenerating their organ tissues.

They are also used in drug discovery.[10] GM zebrafish are being explored for benefits of unlocking human organ tissue diseases and failure mysteries. For instance, zebrafish are used to understand heart tissue repair and regeneration in efforts to study and discover cures for cardiovascular diseases.

Transgenic rainbow trout (*Oncorhynchus mykiss*) have been developed to study muscle development. The introduced transgene causes green fluorescence to appear in fast twitch muscle fibres early in development which persist throughout life. It has been suggested the fish might be used as indicators of aquatic pollutants or other factors which influence development.

In intensive fish farming, the fish are kept at high stocking densities. This means they suffer from frequent transmission of contagious diseases, a problem which is being addressed by GM research. Grass carp (*Ctenopharyngodon idella*) have been modified with a transgene coding for human lactoferrin, which doubles their survival rate relative to control fish after exposure to Aeromonas bacteria and Grass carp hemorrhage virus. Cecropin has been used in channel catfish to enhance their protection against several pathogenic bacteria by 2–4 times.

Transgenic Chickens

Chickens

- grow faster than sheep and goats and large numbers can be grown in close quarters;
- synthesize several grams of protein in the "white" of their eggs.
- Two methods have succeeded in producing chickens carrying and expressing foreign genes.
- Infecting embryos with a viral vector carrying
- the human gene for a therapeutic protein
- promoter sequences that will respond to the signals for making proteins (e.g. lysozyme) in egg white.
- Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.
- Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay.

Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins — something that E. coli cannot do.

Applications of transgenic animals:

Transgenic animals have potentially broad application for the improvement of animal production quality, the enhancement of production capacity, the studies of human disease models and the production of biomedical materials.

The benefits of these animals to human welfare can be grouped into the following areas:

Agricultural applications

The application of biotechnology to farm animals has the potential to benefit both humans and animals in significant ways.

a. Breeding: Farmers have always used selective breeding to produce animals that exhibit desired traits (e.g., increased milk production, high growth rate). Traditional breeding is a time-consuming, difficult task. When technology using molecular biology was developed, it became possible to develop traits in animals in a shorter time and with more precision. In addition, it offers the farmer an easy way to increase yields. Take ES cell technology as an example, chimeric nuclear transfer technology and production technology is improving, as ES cells are widely used in animal cloning. Proliferation of ES cells derived from donor as the nucleus, produced cloned animals. ES cells in germline chimeric, then develop into sperm or eggs to produce offspring. Animal cloning technology can produce excellent breeding, combination of genes and their high proportion in the population in short time.

b. Quality: Transgenic cows exist that produce more milk or milk with less lactose or cholesterol, pigs and cattle that have more meat on them, and sheep that grow more wool. In the past, farmers used growth hormones to spur the development of animals but this technique was problematic, especially since residue of the hormones remained in the animal product. At present the production of transgenic animals in low efficiency is one of the main problems. The results of the testing work are carried out at the individual level. Using ES cells as a carrier, directed transformation of ES cells, the integration of inserted genes, expression level and stability of

interested genes can be screened. The work is carried out at the cellular level, which is easy to obtain stable cell line with expression of satisfaction, accessing to the target gene carrying the transgene for animals. One success story is artificial insemination: the use of this technology from 1950s to 1990s in US, increased the average milk production per cow over 300%.

Medical applications

a. Xenotransplantation: Transplant organs may soon come from transgenic animals. Transgenic pigs may provide the transplant organs needed to alleviate the shortfall. Currently, xenotransplantation is hampered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein. For organ and tissue transplantation, which is known as a "species of daughter cells ", for the clinical organization, organ transplantation offers great amount of material knockout cells. U.S. ACT companies put the nucleus of human skin into bovine oocytes without the genetic information, nurturing issued totipotency cell. If they could be successfully used in clinical, in future, many difficult diseases such as Parkinson's disease will be cured.

b. Nutritional supplements and pharmaceuticals: Milk-producing transgenic animals are especially useful for medicines. Products such as insulin, growth hormone, and blood anti-clotting factors may soon be or have already been obtained from the milk of transgenic cows, sheep, or goats. Research is also underway to manufacture milk through transgenesis for treatment of debilitating diseases such as phenylketonuria (PKU), hereditary emphysema, and cystic fibrosis. ES cell culture techniques are used in some special body, then the cost can be a huge improvement. For example, some special drugs (interferon, antithrombin, erythropoietin and other biological systems agents or genetically modified), in body fluids from animals (milk, blood, etc.) or tissue extract achieve the body of the animal drug production factory.

c. Human gene therapy: A transgenic cow exists that produces a substance to help human red cells grow. Human gene therapy involves in adding a normal copy of a gene (transgene) to the genome of a person carrying defective copies of the gene. The potential for treatments for the 5,000 named genetic diseases is huge and transgenic animals could play a role. The most current human serious medical diseases are cancer, genetic diseases, including birth defects, These

diseases are caused by abnormal cell transformation and differentiation, such as Lesch, Nyhan. Fully understanding the process of cell differentiation and development will be able to cure the diseases. Many scientists have established many mouse disease models, and expressed human disease gene in mice for further treatment of human disease. For example, U.S. National Institute of Molecular Neurology Laboratory used mice ESC to induce neuroepithelial cells, implanted them into the brain, and got a large number of small conflicts like cells and glial cells. It can be envisaged to treat multiple sclerosis diseases.

Ethical issues of animal biotechnology

James Watson and the late Francis Crick discovered the structure of DNA in 1953; Watson has spoken in favour of genetic engineering

Biotechnology isn't something new - selective breeding to create more useful varieties of animals and plants is a form of biotechnology that human beings have used for thousands of years.

Biotechnology includes any use of science or technology to alter the characteristics of a particular breed or animal.

Biotechnology can be good or bad for animals - and it may also produce an answer to the ethical problems of experimenting on animals.

Transgenic animals raise a particularly difficult problem.

Human problems

Newspaper articles about the ethical problems of genetically engineered animals are usually concerned about the danger these animals may pose to human beings (usually to human health), rather than any implications for the animals themselves.

Animal rights

Genetic engineering and selective breeding appear to violate animal rights, because they involve manipulating animals for human ends as if the animals were nothing more than human property, rather than treating the animals as being of value in themselves.

Recent action to allow animals to be patented reinforces the idea of animals as human property, rather than beings in their own right.

Animal welfare

Biotechnology can be good for animals. Selective breeding and genetic engineering can benefit animals in many ways:

- Improving resistance to disease
- Breeding to remove characteristics that cause injury
    - eg selecting cattle without horns

But biotechnology can also be bad for animals - the good effects for the breeder can offset by painful side-effects for the animals:

- Modern pigs have been bred to grow extra fast - some breeds now grow too fast for their hearts, causing discomfort when animals are too active
- Broiler chickens are bred to grow fast - some now grow too fast for their legs

Regulating genetic engineering

Profitability is one of the major drivers of both selective breeding and genetic engineering.

If animal welfare is not to be compromised, research must be restricted by a counter-balancing ethical principle that prevents altering animals in a way that was bad for the animal.

One writer, Bernard Rollin, suggests that a suitable rule to regulate genetic engineering would be this:

Genetically engineered animals should be no worse off than the parent stock would be if they were not so engineered.

This principle can easily be adapted to cover selective breeding.

Biotechnology and experimental animals

It's been suggested that genetic engineering may solve all the ethical problems of laboratory experiments on animals. The goal is to create a genetically engineered mammal that lacks sentience, but is otherwise identical to normal experimental animals.

Such an animal could not suffer whatever was done to it, so there should be no ethical difficulty in performing experiments on it.

Ethical problems:

- This argument seems convincing, but do you feel comfortable about it?
- Is there any ethical objection to creating genetically engineered human beings without sentience, and experimenting on them?

Transgenic animals

Less controversially, scientists are reconstructing the quagga - which became extinct in the 1870s

Transgenic animals are animals that have been deliberately bred for research and that contain elements of two different species - they are creatures that blur the barrier between species.

These animals are often deliberately created with genetic defects, and these defects may well cause the animal to have a bad quality of life. A mouse has been created, for example, that has been genetically modified to develop cancer.

Ethical issues of transgenic animals

Transgenic animals raise several particular moral issues (quite apart from any damage they might do to the environment):

- Are animals that combine species an unethical alteration of the natural order of the universe?
- Is it unethical to modify an animal's genetic make-up for a specific purpose, without knowing in advance if there will be any side-effects that will cause suffering to the animal?

- Does 'creating' animals by genetic engineering amount to treat the animals entirely as commodities?
- Is it unethical to create 'diseased' animals that are very likely to suffer?
    - Suffering may last for a long time in these animals as researchers want to conduct long-term investigations into the development of diseases

Religious views of transgenic animals

Against transgenic animals:

- God laid down the structure of creation and any tampering with it is sinful.
- Manipulating DNA is manipulating 'life itself' - and this is tampering with something that God did not intend humanity to meddle with.

In favour of transgenic animals:

- As human beings have been given 'dominion' over the animals, they are entitled to tamper with them.
- Palaeontology shows that the structure of creation has changed over time as some species became extinct and new ones came into being. They say that this shows that there is nothing fixed about the structure of creation.

Transgenic animals and religious food laws

Transgenic animals pose problems for religions that restrict the foods that their believers can eat, since they may produce animals that appear to be one species, but contain some elements of a forbidden species.

Possible Questions

2 Marks

1. What is transgene
2. Define Transgenesis.
3. What is Xenotransplantation.

8 Marks

1. Explain in detail production of transgenic mouse
2. Briefly explain DOLLY
3. With examples, discuss Gene Therapy
4. Explain the production of transgenic sheep
5. Write in detail about production of transgenic fish
6. Briefly explain the applications of transgenic animal
7. How can a foreign gene can be inserted in cow? Explain
8. Brief about transgenic Pig.
9. Discuss about the ethical issues of animal biotechnology.

Unit 1	Opt 4	Opt 1	Opt 2	Opt 3	Answer
Animal cells have pH…….	8-8.5	7-7.4	5-5.5	6-6.5	7-7.4
What.is used as pH indicator in ATC	Phenol red	Phosphate buffer	Salt sodium	Saline solution	Phenol red
What temperature is maintained for cold blooded animals in the cell culture	30-40°C	15-25 °C	30-35°C	40-45°C	15-25 °C
Glutamate is a key molecule in cellular	All cellular function	Respiration	Metabolism	Digestion	Metabolism
Cells require attachment for growth is called	Adhesive	Anchorage dependent	Matrix dependent	Non adhesive dependent	Anchorage dependent
Animal tissues chopped up to ---------- pieces for explant preparation	2mm2	1mm	0.1mm	0.01mm	1mm
Which of the following is not a serum constituent	Mg and Cu	Binding proteins	Cholesterol	Fe and Zn	Mg and Cu
In animal cell culture phenotypic stabilization requires ---------- days	8 days	5 days	6 days	7 days	7 days
pH of HEPES is ----------	7.4 to 7.6	7 to 7.2	7.2 to 7.4	7.2 to 7.6	7.2 to 7.6
The albumin present in serum do not carries-------	sugar	Lipids	Minerals	Globulins	sugar
_____is present only in fetal serum and it enhances cell attachment.	Antitrypsin	Fetuin	Transperin	Fibronectin	Fetuin
Fetuin is present in…..	Liver cells	fetal serum only	Human serum	Heart cells	Human serum
Which of the following helps to detoxify free radicals as a co factor for GSH-------- synthetase	Serum proteins	Zinc	Selenium	Copper	Selenium
Eagle's MEM supplemented with calf serum helps to maintain ----------	Melanocytes	Human diploid fibroblasts	Skeletal muscles	Neurons	Human diploid fibroblasts
Which of the following is the selection medium for 3T3 cells?	MEM with FB	MEM with CS	M199 with FB	DMEM with CS	MEM with CS
Serum is heat inactivated by incubating it for ----------	30min at 760C	15min at 370C	15min at 560C	30min at 660C	15min at 560C
_____used to promote cell migration from explants	Calf serum	Plasma clot	Suspention culture	Rolling bottles	Plasma clot

Which of the following substance mitogenic to fibroblast cells	TPGF	TGF	PDGF	MDCK	PDGF
Which enhances plating efficiency of different cell types	Fibronectin	Somatotrophin	carticosterone	Fibroin	Somatotrophin
Hepatocyte growth factor is morphogenic to------	haematopoietic cells	Hepatocytes	Kidney tubules	keratinocytes	Kidney tubules
In the absence of serum, the plastic substrate needs to be coated with	phorbol	Polylysine	oncostatin	transferrin	Polylysine
Usual volume of medium to surface is….	0.4 -0.5ml/cm2	0.1 -0.2ml/cm2	0.2 -0.3ml/cm2	0.3 -0.4ml/cm2	0.2 -0.3ml/cm2
Gaseous diffusion become limited if medium is	c Greater than 7 mm	Greater than 1 mm	Greater than 3 mm	Greater than 5 mm	Greater than 5 mm
Media with reduced serum concentration is called as	Minimal media	Holding media	Serum free media	Complex media	Serum free media
Exhaustion of the media is in which phase of the growth curve	Log	Lag	exponential	plateau	exponential
In animal tissue culture gaseous equilibrium maintained in a	O2 incubator	Co2 incubator	Liquid nitrogen container	Aerated carrels flask	Co2 incubator
_____act as a major source of animal tissue contamination	Aerated incubator	CO2 incubator	Humidified incubator	O2 incubator	Humidified incubator
Which is the boiling point of liquid nitrogen	-70°C	-196°C	-96°C	-80°C	-96°C
Which are the cryoprotectants to freeze "spare" expanded human blastocysts	Chloramphenicol	Glycerol and sucrose	Glycerol and glutamine	Progesterone and sucrose	Glycerol and sucrose
After trypsinization, which should be added to neutralize enzyme activity?	EDTA with serum	Medium with serum	BSS with serum	Cold PBS	Medium with serum
The inner surface of the culture dish s typically coated with mouse embryonic skin cells that have been treated so that they will not divide is called ----------	Epithelial cells	feeder layers	monolayers	Nutrient matrix	feeder layers
Which of the following is not coming under enzymatic tissue disaggregation in animal cell culture?	Protease	Pronase	Collagenase	Vernase	Protease

_____are mainly used for production of cell suspensions from established cultures of epithelial type.	Pronase	EDTA	Trypsin	Collagenase	EDTA
Trypsin disaggregation will damage---------- --	B cells	epithelial cells	fibrous tissues	Bone cells	epithelial cells
Trypsin disaggregation will ineffective in ----------	T cells	Epithelial cells	fibrous tissues	Bone cells	fibrous tissues
The technique used for the preservation of germplasm at temperature below sub zero degree is known as……..	Dehydration	Cryopreservation	Freezing	Sublimation	Cryopreservation
Few newly acquired cell stored in ampule is called	Free suspension	Token freeze	Seed stock	User stock	Token freeze
Ampoules are made of ---------	Glass	Polyethylene	Silicon	Nylon	Silicon
Which of the following is used for cryopreservation?	Sodium Cloride	Nitrogen gas	Glutamine	DMSO	DMSO
3T3 cells are used in the cultivation of ----------	hepatic cells	blood leucocytes	stem cells	keratinocytes	keratinocytes
Following is not a physical tissue disaggregation…..	forcing through proteins	forcing through cheese	forcing through silk cloth	forcing through glass beads	forcing through proteins
Minimum liquid ambient require for animal tissue culture is	200 - - 1960	40 - 700	20 - 200	40 - 200	40 - 200
Who discovered tumor cell lines?	Cn Leach	George and Margaret Gey	Enders	Avery Hill	George and Margaret Gey
High carbonate and 5% CO2 is the concentration of ---------- BSS.	hames's	earle's	hank's	eagle's	earle's
Calcium is reduced in suspension culture in order to	Both a and b	maximize cell aggregation	maximize cell attachment	maximize cell proliferation	Both a and b
Accumulation of lactic acid in animal tissue culture medium implies--------	none of the above	improper citric acid cycle	improper glycolysis	both a and b	improper citric acid cycle
Transformed cells derived from a single parental cell are called…	Finite cells	Hybrid line	Cell line	Infinite cells	Cell line
_____Providing a large surface area to cells in small volumes of media.	None of the above	Stirred reactors	Microcarriers	Bath a and b	Bath a and b

Commercially available Microcarreirs are not made from	Glasswool	DEAE Sephadex	Cellulose	Glass and gelatin	glass wool
Microcarriers, macrocarriers or encapsulated beads can not be used in	Solid culture	Fixed-bed reactors	As immobilised matrix	The culture fluid is circulated in a closed loop	solid culture
Hollow fiber reactor consist of ---------- it helps "fibers", cells to grow	Metabolic by-products	Semi-permeable membranes	Definite cut-off cells	Nutrients into the lumen	Semi-permeable membranes
Osmolarity of animal cell is around…..	350-390 mosm/kg	290-300 mosm/kg	260-320 mosm/kg	320-350 mosm/kg	260-320 mosm/kg
Most commonly used growth factor in serum free media are….	Both a) and b)	Insulin	Hydrocortisone	PDGF	Both a) and b)
Name the chemical used to reduce viscosity in animal tissue culture…..	None of the above	Polyvinyl chloride	Carboxy methyl cellulose	Both a and b	Both a and b
……solution is used to maintain embryo primary mammalian cell culture	Balanced salt solution	Ringer solution	Isotonic solution	Tyrode salt solution	Tyrode salt solution
Function of inhibitor……	Cell separation	Cell attachment	Cell diffusion	Cell proliferation	Cell proliferation
First tissue culture was done by …….	Harrison	Carrel	Baker	White	Harrison
First tissue culture was done in …..	Mice embryo	Chick embryo	Human cells	Frog lymph	Frog lymph
Vitamins are usually sterilized by …..	Steam	Autoclave	Filter	Dry heat	Filter
Unit 2	**Opt 4**	**Opt 1**	**Opt 2**	**Opt 3**	**Answer**
Freezing point of liquid nitrogen……	-80°C	-196°C	-96°C	-296°C	-196°C
Programmed cell death is called as	Haemolysis	Apoptosis	Necrosis	Phagocytosis	Apoptosis
Animal tissues were chopped into ……size for explants preparation	2mm	1mm	0.11mm	0.01mm	1mm
…….is the chelating agent used in tissue disaggregation	None of the above	EDTA	Mg++	Ca++	EDTA
In cold trypsinization, the tissue soaked at….	60 °C	35 °C	50 °C	40 °C	40 °C
Enzymes used in disaggregation	Both a and b	Collagenase	Trypsin	Amylase	Both a and b
Ampules are made up of ……	Silver	Selenium	Aluminium	Silicons	Silicons
Cryocanes are…..	Cans	Cryofreezers	Ampules	Freezer	Ampules
……% of glucose is used in the steps of cryopreservation	20	12	15-Oct	5	15-Oct

Cooling rate of cryopreservation is …..	1 °C/min	2 °C/min	1.5 °C/min	3 °C/min	1 °C/min
In cryopreservation the homebox wall thickness is ….	5mm	20mm	15mm	10mm	15mm
The principle of apoptosis was first described by…….	Andrew wyllie	Karl vogt	Walther flemming	John foxton	Karl vogt
The componenet helps in regulating apoptosis…..	ADP	ATP	Poly ADP ribose polymerase	Poly ATP ribose polymerase	Poly ADP ribose polymerase
In MTT assay number of surviving cells can be determined by……..	Gene expression	Dye reduction	Dye uptake	Metabolite produced	Dye reduction
Which of these helps to detoxify free radicals as a cofactor for GSH…..	Albumin	Zinc	Selenium	Copper	Selenium
……is used to measure cell death in LPH assay	None of the above	Glucate	Lactate	Lactate dehydrogenase	Lactate dehydrogenase
In cell characterization, cells obtained from individuals are known as ……..cells	Autologous cells	Zenogenic cells	Allogenic cells	Isogenic cells	Autologous cells
…. Cells are extensively used in cardiovascular implant.	Autologous cells	Isogenic cells	Zenogenic cells	Allogenic cells	Zenogenic cells
Cells from the body of a donor of a same species are ……	None of the above	Allogenic cells	Isogenic cells	Zenogenic cells	Allogenic cells
In three dimensional culture…..days old mouse embryos used for enamol formation.	17	18	15	10	17
Culturing a part of a organ whole organ invitro is called as …. Culture.	None of the above	Tissue culture	Organ culture	Cell culture	Organ culture
Media used in organ culture……..	None of the above	Liquid	Solid	Liquid and solid	Liquid and solid
……mm slide is used in single coverslip culture	15	10	20	5	20
Organ culture in liquid medium is known as……	Agar gel method	Raft method	Grid method	Raft and grid method	Raft and grid method
In agar gel method ….. parts of 1%agar prepared in BSS.	6	8	9	7	7
……metal is used in grid method of cell culture	Copper	Stainless steel	Silver	Aluminium	Stainless steel

In adult organ culture ……percentage of oxygen is used	85	85	90	95	95
……hours old chick embryos used in embryonic organ culture.	10	30	20	40	40
Doubling time of cells in finite cell culture……	36-72 hours	72 hours	24-48 hours	24-36 hours	24-48 hours
……..colour indicates cell decline during 4th day	Pink	Yellow or orange	Red or pink	Purple	Yellow or orange
The buffer used to remove medium from cells during subculturing of cells.	None of the buffer	Saline buffer	Phosphate buffer	Phosphate saline buffer	Phosphate saline buffer
During subculture, the cells are exposed in ……ml of trypsin.	1	0.2	0.1	0.5	0.1
Single coverslip with plasma clot culture was discovered by……	Adrew nyllie	Carrel	Harrison	Jonh foxtan	Harrison
Freezing point of liquid nitrogen……	-196°C	-96°C	-296°C	-80°C	-196°C
Programmed cell death is called as	Apoptosis	Necrosis	Phagocytosis	Haemolysis	Apoptosis
Animal tissues were chopped into ……size for explants preparation	1mm	0.11mm	0.01mm	2mm	1mm
…….is the chelating agent used in tissue disaggregation	EDTA	Mg++	Ca++	Antibodies	EDTA
In cold trypsinization, the tissue soaked at….	35 °C	50 °C	40 °C	60 °C	40 °C
Enzymes used in disaggregation	Collagenase	Trypsin	Amylase	Collagenase & Trypsin	Collagenase & Trypsin
Ampules are made up of ……	Selenium	Aluminium	Silicons	Silver	Silicons
Cryocanes are…..	Cryofreezers	Ampules	Freezer	Cans	Ampules
……% of glucose is used in the steps of cryopreservation	12	15-Oct	5	20	15-Oct
Cooling rate of cryopreservation is …..	2 °C/min	1.5 °C/min	3 °C/min	1 °C/min	1 °C/min
In cryopreservation the homebox wall thickness is ….	20mm	15mm	10mm	5mm	15mm
The principle of apoptosis was first described by…….	Karl vogt	Walther flemming	John foxton	Andrew wyllie	Karl vogt

The componenet helps in regulating apoptosis…..	ATP	Poly ADP ribose polymerase	Poly ATP ribose polymerase	ADP	Poly ADP ribose polymerase
In MTT assay number of surviving cells can be determined by……..	Dye reduction	Dye uptake	Metabolite produced	Gene expression	Dye reduction
Which of these helps to detoxify free radicals as a cofactor for GSH…..	Zinc	Selenium	Copper	Albumin	Selenium
……is used to measure cell death in LPH assay	Glucate	Lactate	Lactate dehydrogenase	Phosphatase	Lactate dehydrogenase
In cell characterization, cells obtained from individuals are known as ……..cells	Zenogenic cells	Allogenic cells	Isogenic cells	Autologous cells	Autologous cells
…. Cells are extensively used in cardiovascular implant.	Isogenic cells	Zenogenic cells	Allogenic cells	Autologous cells	Zenogenic cells
Cells from the body of a donor of a same species are ……	Allogenic cells	Isogenic cells	Zenogenic cells	genic	Allogenic cells
In three dimensional culture…..days old mouse embryos used for enamol formation.	18	15	10	17	17
Culturing a part of a organ whole organ invitro is called as …. Culture.	Tissue culture	Organ culture	Cell culture	micro culture	Organ culture
Media used in organ culture……..	Liquid	Solid	Liquid and solid	gas	Liquid and solid
……mm slide is used in single coverslip culture	10	20	5	15	20
Organ culture in liquid medium is known as……	Raft method	Grid method	Raft and grid method	Agar gel method	Raft and grid method
In agar gel method ….. parts of 1%agar prepared in BSS.	8	9	7	6	7
……metal is used in grid method of cell culture	Stainless steel	Silver	Aluminium	Copper	Stainless steel
In adult organ culture ……percentage of oxygen is used	85	90	95	85	95
……hours old chick embryos used in embryonic organ culture.	30	20	40	10	40

Doubling time of cells in finite cell culture……	72 hours	24-48 hours	24-36 hours	36-72 hours	24-48 hours
GFP contains …aminoacids	338	238	234	245	238
GFP is a	None of the above	Unstable protein	Stable protein	Toxic protein	Stable protein
…….amino acid is present in the 80th position of GFP	None of the above	Arginine	Glutamine	Alkaline	Glutamine
GFP emits blue light in combination with….	Potassium	Sulphur	Chlorophyll	Calcium	Calcium
Original GFP is isolated isolated from the organism	None of the above	Aquarica vectoria	Acquarica bellucida	Acquarica Virginia	Aquarica vectoria
Size of Aquarica vectoria ranges from ….	10-12 cm/diameter	1-5 cm/diameter	3-5 cm/diameter	5-10 cm/diameter	5-10 cm/diameter
Not an advantages of GFP	Glow in day light	No side effect	Does not affect the function of GOI	Non toxic to cells	Glow in day light
Crystal structure of GFP was solved in…	2012	1996	1991	2002	1996
GFP gets activated with ……..calcium ions.	Two	Three	Five	Six	Three
Gene transfer involves transfer of genes	None of the above	Donor into a recipient	Donor into a donor	Both the above	Donor into a recipient
DNA ligase is used to	None of the above	Join DNA	Separate DNA	Both the above	Join DNA
Uncontrolled growth of cells is called	None of the above	Cancer	Tumor	Both the above	Both the above
Optimum temperature used for extension process in polymerase temperature	None of the above	98°C	72°C	55°C	72°C
Xgal	5-bromo-4-indolyl-3-chloro-?-D-galactoside	5-bromo-4-chloro-3-indolyl-?-D-galactoside	5-chloro-4-bromo-3-indolyl-?-D-galactoside	5-indolyl-4-chloro-3-bromo-?-D-galactoside	5-bromo-4-chloro-3-indolyl-?-D-galactoside
During 1970's … were used in gene transfer	Pig	Cat	Mice	Sheep	Mice
The DNA ligase enzyme isolated from bacteriophage…	T4 DNA Ligase	T3 DNA Ligase	T1 DNA Ligase	T2 DNA Ligase	T4 DNA Ligase
Which is not a part in a recombinant plasmid	co activator	Promoter	Antibiotic resistance gene	Reporter gene	co activator

Adaptors are usually	RNA	DNA Sequence	Protein sequence	Aminoacid sequence	DNA Sequence
How many types of DNA ligases are available?	One	Two	Three	Four	Four
Which enzyme helps to catalyse the polymerization of deoxyribonucleotides into a DNA strand ?	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	DNA polymerase
DNA polymerase III enzyme synthesizes at a rate of _____ nucleotides per second	1000	2000	3000	5000	1000
The enzyme that produces RNA is called	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	RNA polymerase
How many types in DNA polymerases are present in prokaryote?	One	Two	Three	Four	Two
How many types in DNA polymerases are present in eukaryote?	One	Two	Three	Four	Three
Which organism produces Taq DNA polymerase	Thermus aquaticus	E.coli	Bacillus spp	Pseudomonas spp	Thermus aquaticus
In 1990, gene therapy technique was used to treat……disease	Alzheimer's disease	SCID	Diabetes	X-linked inheritance	SCID
LPLD…..	Lipid protein lipase disease	Lipoprotein lipase disease	Lipoprotein lipase deficiency	Lipid protein lipase deficiciency	Lipoprotein lipase deficiency
Adenoviral vector contains……..as a genetic materials	ssRNA	dsDNA	ssDNA	dsRNA	dsDNA
Adeno associated virus is….?	cancer	Toxic virus	Non-toxic virus	None of the above	Non-toxic virus
SCID …..?	Simple combined immune disease	Severe combined immune deficiency	Severe combined immune disease	Simple combined immune deficiency	Severe combined immune deficiency
Somatic cells are..	Sperm cells	Body cells	Sex cells	Ovum cells	Body cells

Vector not used treat gene therapy….	pTZ	Adenoviral vector	Retiroviral vector	Baculoviral vector	pTZ
Retroviral vector contains	dsDNA	ssRNA	dsRNA	ssDNA	ssRNA
ITR ?	Inverted tempered report	Inverted terminal repeat	Inverted tempered repeat	Inverted terminal report	Inverted terminal repeat
Which of the following is not true regarding invivo gene therapy when compared to exvivo gene theraphy ?	Safety check possible	Less invasive	Technically simple	Vectors introduced directly	Safety check possible
The drug for LPLD was approved in	Oct-13	Nov-12	Oct-12	Nov-13	Nov-12
Abbreviate GOI	Gene of interaction	Gene of information	Gene of interest	Gene of interruption	Gene of interest
The tip of the pipette used for microinjection is about …	0.5 to 5.5 µm in diameter	0.5 to 5 µm in diameter	0.5 to 1 µm in diameter	0.5 to 1.5 µm in diameter	0.5 to 5 µm in diameter
The particles used for transformation during particle bombardment	None of the above	Gold	Tungsten	Both the above	Both the above
Liposomes are made up of	None of the above	Protein bilayers	Carbohydrade bilayers	Lipid bilayers	Lipid bilayers
Genetic engineering manipulates gene products at the level of the …	RNA	Protein	Amino acid	DNA	DNA
Palindromic sequences in a DNA	All of the above	Form blunt ends when cut by restriction enzymes	Reflect the same sequences on two sides	Are not useful in recombinant DNA experiments	Reflect the same sequences on two sides
A cDNA version of a gene includes	Both b and c	Codons for a mature mRNA	Sequences corresponding to promoters	Sequences corresponding to introns	Codons for a mature mRNA
Gene targeting is done on a	Early embryonic cell	Sperm	Egg cell	Fertilized ovum	Early embryonic cell
…. Consists of recombinant cells containing different fragments of a foreign genome	Knockout organisms	DNA probes	Homologous recombinants	Genomic libraries	Genomic libraries
…… are used to select gene of interest from a genomic library	Gene targets	Restriction enzymes	Cloning vectors	DNA probes	DNA probes

Which gene transfer technique involves the use of a fatty bubble to carry a gene into a somatic cells	Particle bombardment	Electroporation	Liposome transfer	Microinjection	Liposome transfer
Naked DNA	Contains just the sugar-phosphate bone.	Is free of nucleic acid	Is free of cell	Is free of protein	Is free of protein
Who invented lipofection	None of the above	Gardener	Karl	Bongham	Bongham
How much amount of DNA is injected in microinjection technique	10 picoleter	2 picoliter	4 picoliter	6 picoliter	2 picoliter
In microinjection, the DNA of interest is injected into	None	Fertilized egg	Female pronuclei	Male pronuclei	Male pronuclei
cDNA is obtained from reverse transcription of	none of the above	tRNA	rRNA	mRNA	mRNA
Which of the following is not used as a vector for gene cloning	mycoplasma	plasmid	cosmids	bacteriophage	mycoplasma
Chemical transformants ?	None of the above	Calcium	PEG	Both the above	Both the above
Gutless adeno viruses are also called as….	First generation	Third generation adenoviral vectors	Second generation adenoviral vectors	First generation adenoviral vectors	Third generation adenoviral vectors
Abbreviate GAT..	Genome arrangement therapy	Gene Augmentation Therapy	Gene Arrangement therapy	Gene alignment therapy	Gene Augmentation Therapy
Unit 4	**Opt 4**	**Opt 1**	**Opt 2**	**Opt 3**	**Answer**
……replacement triggers stem cell differentiation	Amino acid and media	Serum	BSS	Feeder layer	Serum
What is fertilization?	Division of cells	The fusion of male and female gametes	The division of the zygote into a larger and larger number of smaller cells.	The development of pattern, shape.	The fusion of male and female gametes
Hollow sphere presenting cell is called ….	Blastomere	Morula	Blastula	Blastocoel	Blastula

The …..grow human embryonic stem cells in the absence of mouse derived "feeder" cell	WiCell research institute	Nature research institute	Renke research institute	All research institute	WiCell research institute
The term for eggs that have only a small amount of yolk that are evenly distributed	Morula	Holoblastic	Telolecithal	Isolecithal	Isolecithal
In which stage of development does a zygote go through the structural and functional specialization of group of cells ?	Fertilization	Growth	Differentiation	Morphogenesis	Differentiation
During gastrulation in vertebrates, the pore created by invagination will become the….	Ear	Brain	Heart	Mouth	Mouth
The fertilized egg undergoes cell division without further growth in the process called…	None	Cleavage	Differentiation	Morphogenesis	Cleavage
Generally only one sperm fertilizes an egg because…	When the first sperm membrane fuses with the egg membrane, it separates the fertilization membrane and forms a barrier to any other sperms.	Sperms are so few that are unlikely to arrive at the same time.	Sperm compete against one another before entering and only the fit one accepted	One small entry hole called the blastopore allows only one sperm to fit through and then seals.	When the first sperm membrane fuses with the egg membrane, it separates the fertilization membrane and forms a barrier to any other sperms.
Which structure in the bird egg corresponds to the egg white used in cooking ?	Nucleus	Albumin	Shell	Yolk	Albumin
Which structure in the bird egg functions to remove the wastes	Rhino virus	Allantois	Albumins	Chorion	Allantois
Unlike the sea star gastrula, the frog does not invaginate, but produces a….	Primitive streak	Blastopore	Graycresent	Blastocoel	Blastopore
Because the fertilized egg of frogs has most of the yolk at the …..pole, it is said to be	Vegetal isolecithal	Animal, telolecithal	Animal, isolecithal	Vegetal, telolecithal	Vegetal, telolecithal

The series of cellular division by which a one cell layered structure becomes a three-layer embryo is known as…	Meiosis	Gastrulation	Cleavage	Blastulation	Gastrulation
Genes that determine how segments develop during morphogenesis are termed ….. genes.	Homeotic	Induction	Cleavage	Differentiation	Homeotic
A homeotic gene codes for a …. Protein, a sequence of 60 amino acids, sets of which determine pattern formation.	Regulatory	Ion	Morphogenetic	Growth	Regulatory
Proteins that influence morphogenesis are….	Trophoblasts	Homebox genes	Mutagens	Morphogenes	Homebox genes
Which stage would show the development of the ectoderm and endoderm germ layers ?]	Cleavage	Gastrulation	Neurulation	Blastulation	Gastrulation
Maternal development is the theory that	Boy and girl fetuses are identified at an early stage.	The sperm determines which genes are turned on and off.	Mother cell cytoplasm is distributed to various cells of the morula and deternines which genes are turned on and off.	Only one sperm in four will locate the true egg rather than the polar bodies.	Mother cell cytoplasm is distributed to various cells of the morula and deternines which genes are turned on and off.
Which statement is not true about homeotic genes ?	They are sequentially arranged in the chromosome in the same sequences they are activated during development of the embrro in drosophila.	They have been found in almost all eukaryotic organisms.	They are the master genes that control all the other genes in the organism.	Each homeotic genes has a variable region and a sequence called homebox.	They are sequentially arranged in the chromosome in the same sequences they are activated during development of the embrro in drosophila.

Spermatogenesis is not directly affected by which hormones…	LH	GnRH	Inhibin	FSH	LH
The most powerful form of the inivitro process is…..	fertilizatin	Intracytoplasmic sperm injection	Intercytoplasmic sperm injection	Cytoplasmic sperm injection	Intracytoplasmic sperm injection
The technique used to conception of a human embryo outside the mother's body	De novo fertilzation	Invitro fertilization	Invivo fertilization	In vitro fetus formation	Invitro fertilization
The best embryo chosen for invitro fertilization is	onecelled	Initial fresh embryos	Fresh embryonic stem cells	Whole embryo	Initial fresh embryos
…..weeks to prepare a female fully and get her ready for egg retrieval.	4 to 8	4 to 6	5 to 6	2 to 3	4 to 6
The first test tube baby was born in the year….	1975	1968	1978	1988	1978
Embryonic stem cells are determined to be…	Pluripotent	Omnipotent	Plastic	Totipotent	Pluripotent
The issue of embryonic stem cell research has generated public debate…	Both a and b.	Embryonic stem cell research could improve human life	Embryonic stem cell research could advance science and medicine	Embryonic stem cell research could destroy human embryos.	Both a and b.
Stem cell research involves the research of … of a human being.	Germinal cells	Primordial cells	Primary cells	Pre embryonic cells	Primordial cells
The use of differentiated stem cells in clinical trials might	Decrease the overall timeline.	Shorten phase I	Reduce the number of animal tests	Shorten phase II	Reduce the number of animal tests
Gametogenesis occur in…	Intestine	Gonads	Egg	brain	Gonads
Gametes are of …. Types	Three	Four	Two	Five	Two
The testis is formed of thousands of minute tubules called as ….	Interstitial tubules	Seminiferous tubules	Secondary tubules	Teritary tubules	Interstitial tubules
Repeated mitotic cell division takes place in…….	Multiplication phase	Growth phase	Maturation phase	decline phase	Multiplication phase
Transformation of the spermatid into spermatozoon is called ….	Spermiogenesis	Oogenesis	Parthenogenesis	Granulogenesis	Spermiogenesis
Acrosomes of the sperm develops from….	Mitochondria	Microtubules	Golgibodies	Graules	Golgibodies

…….is a process by which the ovum develops	Ovulation	Oogenesis	Spermiogenesis	Eugenesis	Oogenesis
Multiplication phase in oogenesis is also called as …….	Growth phase	Maturation phase	Multiplication phase	Developing phase	Multiplication phase
In mammals, the follicle cells and developing ovum together constitute a …..	Liquor follicle	Graffian follicle	Membrane granules	Corona radiate	Graffian follicle
The large nucleus of the oocyte is called…….	Cortical granules	Germinal vesicle	Membrane vesicles	Desosomes	Germinal vesicle
During oogenesis, the oocyte is surrounded by a transparent membrane called…..	Desmosomes	Graffian follicle	Theca externa	Zona pellucid	Zona pellucid
Union of spermatozoon and egg resulting in the formation of zygote is called…..	Gametogenesis	Fertilization	Polysomeogenesis	Euthenics	Fertilization
Fertilization maintains the …. no of chromosomes in the race.	Haploid	Diploid	tetraploid	polyploid	Diploid
The enzymes helpful for the entry of spermatozoa during fertilization…..	Heterogenase	Saccoglossus	Hyaluronidase	Dehudrogenase	Hyaluronidase
During fertilization NAD + ATP is converted to NADP + ADP in the presence of	Kinase	Mutase	Dehydrogenase	Phosphatase	Kinase
The fertilization egg freely rotates inside the …….fluid around fertilization membrane.	Berivitelline fluid	Copulation fluid	Grey crescent	Lactic acid	Berivitelline fluid
Cleavage is initiated by the appearance of a groove or constriction called …..	Cleavage funnel	Cleavage roll	Cleavage bottom	Cleavage furrow	Cleavage furrow
In invitro fertilization, Embryo transfer involves collection ……from donor.	Fertilized ovum	Unfertilized ovum	Sperm	egg	Fertilized ovum
Unit 5					
Gene knockout is a genetically engineered organism that carries	One or more genes in its chromosomes	One or more genes in its cell	One gene of assortment	one gene of seggregation	One or more genes in its chromosomes

Knockout is a route to learning about a gene	That is active	That has been sequenced.	That are suppressed	That are ordered	That are suppressed
Knockout is accomplished through	a combination of techniques	a single technique	Bifunctional technique	mochromatic techniques	a combination of techniques
Knockout require	a plasmid	bacterial artificial chromosome	cell culture	All the three options	All the three options
Gene therapy is the insertion of genes into an individual's	Bones	cells and tissues	Ligaments	Skull	cells and tissues
---------- to treat a disease, and hereditary diseases	Gene therapy	Genetic instability	Genome stability	Transplantation	Gene therapy
A carrier called a ---------- must be used to deliver the therapeutic gene to the patient's	Fusion agent	Trancription initiater	Vector	Illucitor	Vector
The most common type of vector are ---------- that have been genetically altered to carry normal human DNA	PBR322	Cosmids	Viruses	Yeast	Viruses
All gene therapy to date on humans has been directed	somatic cells	Gene level	RNA level	Plasmid level	somatic cells
Somatic gene therapy can be broadly split in to	one category	Five category	Six category	Two categories	Two categories
ex vivo, which means	Cells are modified outside the body and then transplanted back in again	genes are changed in cells still in the body	recombination with a very low probability	Recombination approach	Cells are modified outside the body and then transplanted back in again
vivo, which means	Cells are modified outside the body and then transplanted back in again	genes are changed in cells still in the body	recombinatiorr with a very low probability	Recombination approach	genes are changed in cells still in the body

---------- are the methods to replace or repair the genes targeted in gene therapy	A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common	An abnormal gene could be swapped for a normal gene through homologous recombination	The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function	All the three options	All the three options
The genetic material in retroviruses is in the form	DNA molecule	RNA molecules	Proteims	Lipids	RNA molecules
Antisense refers to short	DNA sequences	RNA sequences	Both DNA and RNA sequences	Any chemical substances	Both DNA and RNA sequences
--------- are designed to be complementary to a specific gene sequence to inhibit activity	Oligonucleotides	Gene fragments	Similar sequences	Antisense oligonucleotide	Antisense oligonucleotide
In principle, antisense technology is supposed to prevent	Protein production from a targeted gene	Amino acid synthesis	Mutate cells	Elongate the cell cycle	Protein production from a targeted gene
RNA interference is a mechanism	DNA -guide regulation of gene expression	RNA-guided regulation of gene expression	Reverse trancriptase -guided regulation mechanism	Conserved pathway modifiction	RNA-guided regulation of gene expression
In RNAi_____ inhibits the expression of genes	Viruses	Double stranded DNA	double-stranded ribonucleic acid	Bacterial DNA	double-stranded ribonucleic acid
RNAi pathway is thought to have evolved as a form of	innate immunityagainst viruses	Innate immunity against protozao	Innate immunity against bacteria	Innate immunity against fungi	innate immunityagainst viruses
The RNA interference pathway is often exploited in experimental biology	To study the activation of genes	To study the biology of gene	to study the function of genes in cell culture and in vivo in model organisms.	To study the history of genes	to study the function of genes in cell culture and in vivo in model organisms.

Vaccines that use components of a pathogenic organism rather than whole organism is called	Peptide vaccines	Triplet vaccines	MAB	Subunit vaccines	Subunit vaccines
Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as	knock out	knock Down	knock up	Knock in	knock Down
In subunit vaccine for Herpes simplex virus are cloned into	Chines hamster ovary	Lung cell lines	Mamalian cell lines	Hela cell lines	Chines hamster ovary
Which of the following is not a type of animal patent?	Landrace	Gain of function (transgene)	Loss of function (knockout)	Bioreactors	Landrace
Due to lack of ….. animal cells appear to be circular	cellulose	Cell wall	Nucleus	Cellulose	Cell wall
Insertion of genes into eggs of animals is carried out by …… method	transformation	Micro-injection	Electroporation	Micromanipulator	Micro-injection
Preproinsulin is synthesized in the ----------cells of pancreas	Transformation	α cells	β cells	γ cells	α cells
Swine insulin is transformed in to human insulin by replacing the	Valine residue	alanine residue	Cysteine residue	Guanine residue	alanine residue
Sendai viral capsid stimulate human chromosome to produce----------	Immunoglobulin G	Interferon	Interleukin	Immunoglobulin M	Interferon
Interferon γ is produced by sensitized lymphocytes in response to the----------	Both a and b	Sensitizing antigen	Non-specific antigens	Specific antigens	Both a and b
Human peptide hormone synthesized in bacterial cell was	somatostatin	Estrogen	secretin	scmatoliberin	somatostatin
Productioln of active somatostain requires ---------- to cleave β galactosidase from inactive somatostatin	None of the above	Cyauaogcn bromide	Growth hormone gene	Restriction endonucleases	Cyauaogcn bromide
β- propiolactone inactivated virus in embryonated duck eggs used to develop----------	Tetanus vaccine	Rubella vaccine	Rabies vaccine	Plague vaccine	Rabies vaccine
-------is absent in humans causes the inherited disease ovteogenesis imperfecta	COLA BI gene	COLLIAI gene	AIADAI	Alpha AI gene	COLLIAI gene

Transgenic ---------- express large quantities of the human alp hal-antitrypsin protein in their milk	Both band c	Cow	Sheep	embryo splitting	Both band c
which Serve as a source of transplanted organs for humans	Transgenic sheep	Transgenic pigs	Transgenic rabbits	Transgenic goats	Transgenic pigs
Porcine endogenous retrovirus is ----------	Human retrovirus	Pig retrovirus	Bovine retrovirus	Goat retrovirus	Pig retrovirus
Retroviral method of DNA transfer successfully used in 1974 when a simian virus was inserted into ----------	Rodent embryos, resulting in mice carrying this DNA	Mice embryos, resulting in mice carrying this DNA	Rabbit embryos, resulting in mice carrying this DNA	Pig embryos, resulting in mice carrying this DNA	Mice embryos, resulting in mice carrying this DNA
1n 1997, the first transgenic cow, Rosie, produced human protein-enriched milk at ----	4.4 grams per liter	1.4 grams per liter	2.4 grams per liter	3.4 grams per liter	2.4 grams per liter
The first death of participant in gene therapy experiment is ----------	None of the above	Jesse Gelsinger	ames Wilson	Mark Batshaw	Jesse Gelsinger
Chimerus comprise a mixture of cells from two or more	Both a and b	Genetically distinct organisms of the same species	Organisms of different species	They are mosaics at the sexual level	Both a and b
---------- Carrying a gene that promotes the development of various human cancers	none of the above	Harvard mouse	On co mouse	Both a and b	Both a and b
Small pox vaccines was first discovered by	Robert hooke	Loulis Pasteur	Paul ehlrich	Edward jenner	Loulis Pasteur
Fusion of rat b cells with mouse myeloma cells are called as	a;l the above	Interspecific clone	interspecies hybridoma	hybrid cell	Interspecies hybridoma
Transplantation of heart valve from pig to human is example for	zoograft	Allograft	Xenograft	Isograft	Xenograft
Tetanus vaccine is an	cellular vaccine	Inactivated vaccine	Toxoid vaccine	Recombinant vaccine	Toxoid vaccine
Live attenuated vaiccine was first introduced by	Robert Hooke	Louis pasteur.	Paul Ehlrich	Edward Jenner	Louis pasteur.

The day of detection of a mouse vaginal plug is called as	none of the above	Dating the embryo	embryo harvesting	estrus induction	Dating the embryo
Optimal age for whole embryo preparation is	20 days	10 days	13 days	16 days	13 days
In *in vivo* condition tumor necrosis starts from the	In the medium	Outer layer	Middle layer	Inner layer	Outer layer
Experimental liver modeling can be obtained by	Embryo culture	Spheroids co culture	heterotypic 3D culture	Both a and b	Both a and b
Oxygen regulated gene expression during embryonic development studied	Stem cell Culture	Three dimentional culture	Organ culture	Tissue culture	Three dimentional culture
Three dimensions of the animal cells are normally ……..in shape	Spherical	Round	Polygonal	Rectangular	Spherical
Due to the lack of ---------- animal cells appear to be circular	membrane	Cell wall	nucleus	cellulose	Cell wall
Which of the following is the cytoskeleton of animal cell	Myosin	Actin filaments	Mititic filaments	micro tubules	Actin filaments
BCG is a ----------	Attenuated vaccine	Toxoid vaccine	Recombinant vaccine	Cellular Vaccine	Attenuated vaccine
An example for whole organism as vaccine	Tetanus	Tuberculosis	Diphtheria	Hepatitis	Tuberculosis
In DNA vaccine technology ---------- injected directly to the muscle	Plasmid DNA	DNA fragments	Genomic DNA	RNA	Plasmid DNA
Which among the following statement is false?	DNA vaccines induces response to a multiple epitope	Attenuated vaccine induce cell mediated immunity	proteins	DNA vaccines do not generate immunologic memory	DNA vaccines do not generate immunologic memory
Source of methionine for methylation obtained from	S- Adenosyl Methionine	Methionine	Protein synthesis	Transcription	S- Adenosyl Methionine
PoIynucleotide kinase involves	Transfer of Hydroxyl group to the terminal end of DNA	Transfer of terminal phosphate group from ATP	Promotes for transfer	Prevents the transfer	Transfer of Hydroxyl group to the terminal end of DNA

DNA Polymerase I Holoenzyme are	Single functional enzyme	Bi functional enzyme	Multifunctional enzyme	co enzymes	Bi functional enzyme
A Polymerase Klenow fragment	Posses Polymerase activity	Posses 5" exonuclease activity	Lack exonuclease activity	Alkaline phosphatase	Posses Polymerase activity
Oxygen regulated gene expression during embryonic development studied	Three dimentional culture	Organ culture	Tissue culture	Stem cell Culture	Three dimentional culture
Due to the lack of ---------- animal cells appear to be circular	Cell wall	nucleus	cellulose	DNA	Cell wall
Which of the following is the cytoskeleton of animal cell	Actin filaments	Mitotic filaments	micro tubules	vesicles	Actin filaments