TOPIC P: APPLICATIONS OF MOLECULAR AND CELL BIOLOGY

Learning Outcomes

Application Topic 9: Application of Molecular and Cell Biology

Candidates should be able to

(a) Describe the unique features of zygotic stem cells, embryonic stem cells and blood stem cells; correctly using the terms:

- Totipotency

  Zygotic stem cells which have ability to differentiate into any cell type to form whole organisms and so are also pluripotent and multipotent.

- Pluripotency

  Embryonic stem cells which have ability to differentiate into almost any cell type to form any organ or type of cell and so are not totipotent but are multipotent.

- Multipotency

  Blood stem cells which have ability to differentiate into a limited range of cell type and so are not pluripotent or totipotent.

(b) Explain the normal functions of stem cells in a living organism (e.g. embryonic stem cells and blood stem cells).

(c) Describe:

- two types of genetic diseases e.g. SCID (severe combined immunodeficiency) and cystic fibrosis;
- the treatment of SCID and cystic fibrosis, using viral and non-viral gene delivery systems respectively. (Details of manipulation of genes and formation of vectors carrying genes are not required.)

(d) Explain the factors that keep gene therapy from becoming an effective treatment for genetic diseases.

(e) Discuss the social and ethical considerations for the use of gene therapy.

(f) Discuss cloning in plants in terms of plant tissue culture techniques (Gene cloning in plants using *Agrobacterium* spp. and gene gun are not required.)

(g) Explain the significance of genetic engineering in improving the quality and yield of crop plants and animals in solving the demand for food in the world (e.g. Bt corn, golden rice, GM salmon).

(h) Discuss the ethical and social implications of genetically modified crop plants and animals (e.g. Bt corn, golden rice, GM salmon).

Use the knowledge gained in this section in new situations or to solve related problems.

References

1. Campbell N. A. and Reece J.B. (2005). Biology. Chapter 20: DNA Technology and Genomics. Seventh Edition. Pearson Education, Inc.
2. Snustad D. P., Simmons M. J., Jenkins J. B. (1997). Principles of Genetics. Chapter 20: Molecular Analysis of Genes and Gene Products. First Edition. John Wiley and Sons, Inc.
3. Plant Propagation by Tissue Culture 3rd Edition. (2008). Vol. 1. M. A. H. Edwin F. George, Geert-Jan De Klerk (Ed.) *Volume 1. The Background*
4. Websites related to cloning in plants, its significance, and social and ethical issues:
   (a) http://edugreen.teri.res.in/explore/bio/tissue.htm
   (b) http://www.ornl.gov/sci/techresources/Human_Genome/publicat/primer2001/7.shtml
   (c) http://bioethicsweb.ac.uk/browse/mesh/D030841.html
   (d) http://fpc.state.gov/fpc/6176.htm
   (e) http://www.cimmyt.org/abc/10-faqaboutgmos/htm/10-faqaboutgmos.htm
5. Websites related to gene therapy, and its social and ethical issues:
   (a) http://www.scid.net/about.htm
   (b) http://www.ygyh.org/cf/whatisit.htm
   (c) http://www.ornl.gov/sci/techresources/Human_Genome/medicine/genetherapy.shtml#factors
   (d) http://www.ornl.gov/sci/techresources/Human_Genome/publicat/primer2001/6.shtml
   (e) http://www.ornl.gov/sci/techresources/Human_Genome/publicat/primer2001/8.shtml
6. Websites related to stem cells
   (a) http://www.exploratorium.edu/imaging_station/gallery.php?Category=stemcells
   (b) http://stemcells.nih.gov/info/basics/basics1.asp

Content Outline

1. Introduction
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   (b) Genetic engineering in Plants
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5. Gene therapy
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   (d) Social and Ethical Issues

1. Introduction

In the previous topic, we have covered how recombinant DNA can be made using various molecular techniques. In this topic, we will focus on the application of genetic engineering technologies to plants and animals. Some of these applications include:

- Introduction or enhancement of desirable traits in agronomic plants and domestic animals
- Production of valuable products by genetically modified plants and animals
- Investigation of basic biological processes such as gene expression in genetically modified organisms
- Gene therapy and stem cell research.

A **genetically modified organism** is an organism that has its genetic material altered through the use of **recombinant DNA technology**. This technology involves the combination of DNA molecules from different sources into one molecule to create a new set of genes.

A **transgenic** organism, a specific type of genetically modified organism, is an organism that has its genetic material altered by the insertion of a transgene, which confers desirable traits, through the use of recombinant DNA technology.

The **transgene** is a DNA molecule obtained from **different species**, and can be **inherited to subsequent generations**. DNA is taken up by the cells and then inserts itself into one or more of the recipient's chromosomes; it is then inherited like any other gene. From a transgenic cell, a multicellular **transgenic organism** can be produced; all of whose cells contain the transgene.

2. Techniques

Plants have been genetically manipulated by plant breeders for decades. Today, plant breeders can directly modify the DNA of organisms and introduce genes from other species to plant or animal genomes by recombinant DNA techniques. To date, scientists have successfully manipulated various plants and animals to possess phenotypically beneficial characteristics.

(a) Plant Tissue Culture

Plant tissue culture is the technique of growing pieces of plant cells, tissues or organs (explants) **isolated from the source plant**, under controlled conditions on **sterile artificial medium**. This method of growing plants is called *in vitro* growth (outside the organism) as opposed to *in vivo* (within the organism).

Micropropagation is used specifically to refer to the use of tissue culture technique to propagate plants. The objective of micropropagation is to create **clones**, via **asexual propagation**. This is especially useful in regeneration of genetically engineered plants and genotype modification, in which many exact copies are needed.

(i) Plant Tissue Culture (Micropropagation) Procedure

Preparation of the culture medium

The plant tissue culture media supply the nutrients necessary for growth. However, the components of the nutrient medium may differ depending on factors such as the species of plant to be cultured, type of organ cultured and stages of culture. The most common medium used is Murashige and Skoog medium (MS medium).

- Inorganic mineral elements, which include macronutrients and micronutrients
- Macronutrients: Salts of nitrogen, potassium, magnesium, calcium, phosphorus, sulphur
- Micronutrients: Salts of iron, zinc, manganese etc. This is needed in much lower concentrations than macronutrients.
- Organic compounds:
  - Sugar (e.g. sucrose; as a carbon or energy source)
  - Vitamins (cofactor for metabolic functions)
  - Amino acids
- **Plant growth regulators**: E.g. Auxins and Cytokinins. Auxin and Cytokinin ratio will affect the morphogenesis of cultured tissues
  - **High cytokinin to auxin ratio (cytokinin > auxin) : Shoot formation**
  - **High auxin to cytokinin ratio (auxin > cytokinin) : Root formation**
- Solidifying agent: Agar is used to solidify tissue culture media into a gel
- Other substances such as **antibiotics or herbicides** are added for selection of successfully transformed cells.

Stage 1: Establishment of aseptic culture

- An **explant** is the part of a plant (tissue / organ) which is selected to grow or culture. A healthy / disease-free plant is selected. A small section of the shoot, root or leaf will usually be used because they contain **meristematic cells** which are able to divide and differentiate into any other type of cell. This feature is known as **totipotency**.
- Preferred explant: shoot tip, axillary bud.
  - Explant sources can also include embryos, anthers
  - Very often, a **protoplast cell**, which is a plant cell that has its cell wall removed, is used.
- The explant is **surfaced sterilized using alcohol or sodium hypochlorite**, before the transformed tissue is **transferred aseptically** to medium agar, consisting of nutrients and plant growth hormones. This ensures that they are free from microbial contaminants.
- Vessels and media in which the cultures are grown, apparatus such as forceps and scalpels also have to be sterilized. All preparations are performed in the sterile transfer hood.

What is a protoplast culture?

- Protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the **cell wall removed**, bound by cell surface membrane.
- Allows genetic recombination through **somatic hybridisation** in sexually incompatible crosses and also for **plant modification studies**.
  - Somatic hybridisation involves the **fusion** of two distantly related or closely related **plant protoplasts** to create **plant hybrid cells (somatic hybrid)**, which eventually form hybrid plants.
    - Allow breeders to overcome reproductive barriers and combine good traits of two or more plants.
  - Plant modification studies involve the **uptake of DNA** into protoplasts or selective transfer of beneficial gene(s) into protoplasts.
    - Selected DNA fragments can be inserted into the cell, modifying the genetic information of the cell, forming transformed plants.
- **Generates polyploids**, which **improves vigour** in plants (hybrid vigour) i.e. higher yield of fruit, seedless varieties, greater resistance to disease and drought.
- E.g. strawberries, blackberries, potatoes.

Stage 2: Multiplication & Axillary shoot enhancement

- Aim is to obtain maximum number of tissue pieces / clumps for generation of future plantlets.
- Cells of an explant divide by mitosis to form a **callus**, which is a mass of **unorganized** and **undifferentiated** cells.
- As the callus increase in size, pieces of the callus is sliced off and grown on fresh medium in another vessel in a process known as **sub-culturing**. Therefore a callus can be subcultured to become many calli (pl. for callus).
- **Plant growth regulators** are chemical substances produced by plants and they direct growth and differentiation of plants. In the medium, these hormones **direct growth and differentiation of plant tissue.**
- For the formation of callus, **equal ratio of auxin and cytokinin** are added to the nutrient medium.
- By adjusting the concentration of auxin and cytokinin in the growth medium, the cells in the callus can be induced to differentiate into roots and shoots.
- **High cytokinin to auxin ratio (cytokinin > auxin)** → Induce callus to differentiate to produce shoot.

Stage 3: Rooting the shoots obtained from stage 2 culture

- The shoots are re-cultured into a growth medium with **auxin > cytokinin** to induce **root formation**, prior to their transfer to soil.

Stage 4: Acclimatisation (Transfer to natural environment / soil)

- Plantlets containing both roots and shoots are removed from agar medium, agar washed away from roots and **transplanted to sterile soil for further growth in a sheltered environment.** Eventually the young plants can be re-planted in an open uncontrolled environment.
- Allow plantlets to gradually get used to external conditions away from *in vitro* environment where they were previously grown in.
- For e.g. *in vitro* environment, they rely mostly on chemical/ / organic sources of energy (sucrose) and it requires a source of carbon other than CO_2 (unable to produce carbon); chemoheterotrophs. Therefore it is not photosynthetically competent. By placing in external environment, they will gradually become photoautotrophs (derive energy from light, carbon from CO_2).

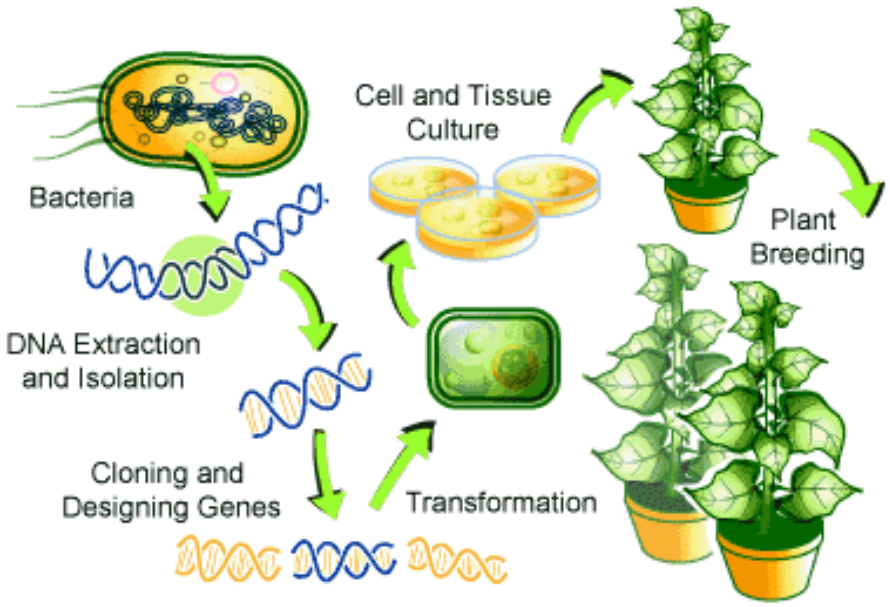


Diagram showing general procedure in plant cloning

(ii) Advantages & Disadvantages of Micropropagation

Advantages:

- **Rapid rate of multiplication** of plants as a single plant can be cloned into many genetically identical copies by subdividing calli as they grow.
- Rate of growth by micropropagation is also greater than conventional propagation as plantlets are grown in a controlled environment, therefore growth is **independent of climatic changes**.
- **Genetic uniformity**; the genetic makeup of all plants possessing desirable phenotypes can be preserved generation after generation.
- Possible to produce clones of some plants species that are otherwise **slow and difficult to propagate** (e.g. **orchid**)
- Able to multiply plants which produce little or no seeds (e.g. **bananas**).
- Production of **disease free plants** when explants are taken from meristematic regions (shoot tips, root tips), which are free from viruses. It is also more difficult for viruses to infect these cells. One possible reason is due to the high metabolic activity in meristematic cells, therefore viruses are unable to take over control of the host biosynthetic machinery.
- Production of new plants can be continued all year round and is **independent of seasonal changes**. Therefore plants can be produced all year round to meet consumer demand.
- **Relatively low space** requirement, as compared to growing plants in greenhouses or farms.

Disadvantages:

- **Contamination of cultures** poses the greatest problem to commercial tissue culture as it can cause very high losses in a short time. Many plant species are susceptible to bacterial, fungal or viral infection, even in very clean environments.
- The **limited gene pool** and **genetic uniformity** of plants cultured make them vulnerable to new diseases or drastic changes in the environment.
- Very **expensive** due to high overhead (specialized equipment, facilities, supplies) and labour costs. It is also labour intensive as labour is required to transfer plantlets from laboratory to soil. Therefore this may not be economical for crops with low financial returns like carrots.
- Plants produced from calli may undergo genetic changes to produce **genetic variations**, which usually result in undesirable phenotypes

(b) Genetic Engineering in Plants

Plant transformation is the process of inserting a **foreign gene** into a plant and having the **gene be expressed and inherited** as part of the plant genome. The result is a genetically engineered transgenic plant.

Methods of insertion of transgenes into plant cells

As plant cells contain the cellulose cell walls, techniques employed must be able to penetrate the plant cell wall. After the gene of interest has been identified, the gene must be introduced into the plant to express the protein. This can be done by:

(i) Electroporation

A short burst of electricity is used to increase permeability of membrane to directly introduce the DNA into plant cell.

(ii) Microprojectile bombardment

DNA-coated tungsten or gold particles are shot into plant cells using a gene gun.

(iii) *Agrobacterium tumefaciens*-mediated transformation

Tumour-inducing (Ti) plasmid is the most common used vector to produce transgenic plants.Plasmid is isolated from soil bacterium, ***Agrobacterium tumefaciens***.

Agrobacterium tumefaciens is a bacterium that causes galls or tumours at the junction between root and stem of dicotyledonous plants. This soil bacterium can infect a plant through any wound and induce tumour formation.

- Ti plasmid contains a **T-DNA** region and **virulence (*vir*)** region.
  - The **T-DNA region** contains genes responsible for the biosynthesis of plant hormones such as auxin and cytokinin. These hormones which stimulates the **formation of tumours**.
  - The ***vir* region** codes for enzymes necessary for the **excision**, **transfer** and **integration** of T-DNA.
  - Under normal circumstances, the bacterium infects a plant cell. The T-DNA is excised from Ti plasmid, transferred into nucleus and integrated into the plant genome.

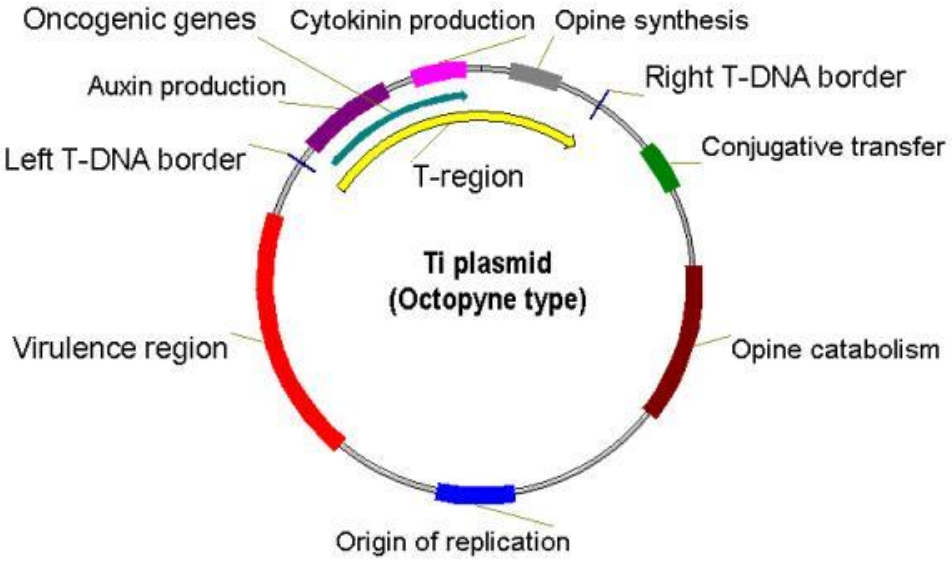
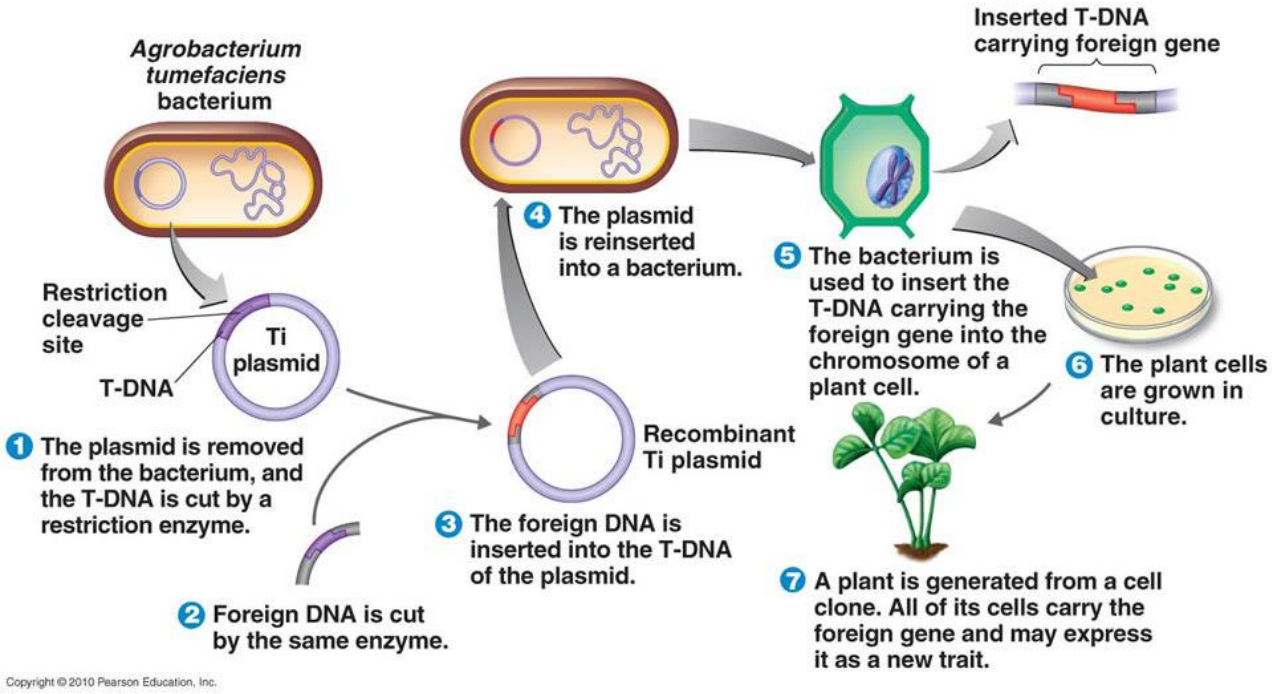


Diagram showing Ti plasmid

- ***Gene transfer via Agrobacterium tumefaciens***

  The ability of integrating its T-DNA into plant genome is made use of by scientists.

  - Tumour-inducing genes / genes encoding biosynthetic genes of **auxin and cytokinin are removed** to disarm T-DNA thus the modified Ti plasmid **does not cause tumour** in plants.
  - **Gene of interest is inserted into the T-DNA region** using restriction enzymes.
  - **Selection marker gene** is also inserted in the recombinant Ti plasmid.
    - Genes that code for antibiotic resistance, or
    - Genes that code for herbicide resistance
  - These selection marker genes allow plant researchers to **select only successfully transformed cells in culture media** containing the antibiotic or herbicide.
  - Recombinant plasmid is then **inserted back into *Agrobacterium***, which is applied as a liquid suspension to the leaves of susceptible plants, infecting them. It can infect susceptible plants or plant cells growing in culture.
  - The recombinant plasmids can be introduced directly into plant cells in plant tissue culture by electroporation.
  - **T-DNA containing the DNA of interest** is integrated into plant chromosome.
  - Plant cells that are successfully transformed would have the antibiotic resistance gene from T-DNA transfer, therefore when placed on medium containing antibiotic, they are able to undergo cell division to form a **callus**.



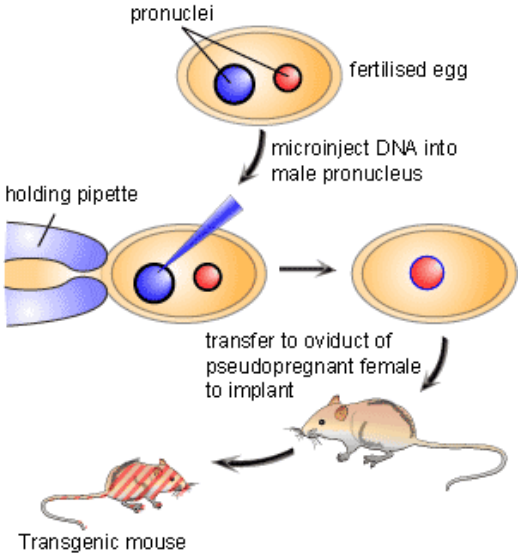
- **Advantage:** Gene of interest can be integrated into the plant genome resulting in stable transgenic plants. The transgene can be inherited in a Mendelian fashion.
- **Disadvantage:** *Agrobacterium* cannot infect most species of monocotyledonous plants (e.g. corn and wheat).

(c) Genetic Engineering in Animals

Two methods are predominantly used to produce transgenic animals:

(i) <u>Microinjection</u> of desirable gene into the pronuclei of fertilized eggs:

- Eggs are surgically removed from female parent and fertilized *in vitro.*
- Desirable gene is microinjected into the male pronucleus (the haploid nucleus contributed by the sperm, prior to nuclear fusion) of the fertilized egg through a very fine-tipped glass needle.
- Injected gene will integrate into the genome at random sites.
- The successfully modified embryo will be identified using selection marker genes.
- The embryo is then implanted into a surrogate female animal for further development.



(ii) Infection of pre-implantation embryos with <u>retroviral vectors</u>:

- Non-virulent retroviruses are used as vectors.
- The gene of interest is inserted into the retrovirus before infection.
- Upon infection, the gene of interest will be integrated into the embryo's genome (*recall: HIV infection*).
- The embryo is then implanted into a surrogate female animal for further development.

3. Genetically Modified Organisms

(a) Importance and Benefits (in improving quality and yield)

As the world population increases at an exponential rate over the recent decades, traditional farming methods are no longer able to sustain produce for the population. Genetic engineering is rapidly replacing traditional plant and animal breeding programs. Useful traits allow plant and animal breeders to obtain a **higher yield** and **better quality** of plant and animal produce.

***Table 1: Examples of GMOs Resulting from Agricultural* Biotechnology**

Genetically Conferred Trait	Example Organism	Genetic Change
APPROVED COMMERCIAL PRODUCTS		
Herbicide tolerance	Soybean	Glyphosate herbicide (Roundup) tolerance conferred by expression of a glyphosate-tolerant form of the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from the soil bacterium *Agrobacterium tumefaciens*, strain CP4
Insect resistance	Corn	Resistance to insect pests, specifically the European corn borer, through expression of the insecticidal protein Cry1Ab from *Bacillus thuringiensis*
Altered fatty acid composition	Canola	High laurate levels achieved by inserting thegene for ACP thioesterase from the California bay tree *Umbellularia californica*
Virus resistance	Plum	Resistance to plum pox virus conferred by insertion of a coat protein (CP) gene from the virus
PRODUCTS STILL IN DEVELOPMENT		
Vitamin enrichment	Rice	Three genes for the manufacture of beta-carotene, a precursor to vitamin A, in the endosperm of the rice prevent its removal (from husks) during milling
Vaccines	Tobacco	Hepatitis B virus surface antigen (HBsAg) produced in transgenic tobacco induces immune response when injected into mice
Oral vaccines	Maize	Fusion protein (F) from Newcastle disease virus (NDV) expressed in corn seeds induces an immune response when fed to chickens
Faster maturation	Coho salmon	A type 1 growth hormone gene injected into fertilized fish eggs results in 6.2% retention of the vector at one year of age, as well as significantly increased growth rates

(i) Improving yield of produce

- **Pest-resistant transgenic plants (E.g. Bt Corn)**

  Pest such as locusts also decreases crop yields. Pesticides have been used extensively to **lower the amount of losses caused by insect damage**. However, recent research has shown that these pesticides have adverse effects on human health. Genetically engineered crops that resist destructive microbes and insects have reduced the need for pesticides.

  - Common soil bacterium ***Bacillus thuringiensis*** possesses a gene which codes for a large crystal-like protein known as the **Bt toxin**.
  - Trangenic plants containing the Bt gene will produce the protoxin which is ingested by the insect.
  - Exposure to high pH (alkaline) in the **insect's gut** caused the **protoxin to be cleaved by enzyme** and the **active Bt toxin is released**.
  - This protein binds to the **specific receptors on the cell membranes** of gut epithelial cells and causes them to be **permeable** causing **gut cells of insect to lyse**, eventually leading to the death of the insects.
  - Bt toxin is not known to have any harmful effects on humans as the acid in our stomach will denature the protein.

- **Herbicide-resistant transgenic plants**

  Weeds compete with crops for soil nutrients and routinely lead to significant losses in yield. Herbicides are used to kill weeds. However they are not specific enough to kill just weeds but will impede the growth of crop plants as well.

  - **Glyphosate** (Roundup™) is one of the most potent herbicide used. It **inhibits an enzyme needed for biosynthesis of essential amino acids** tyrosine, phenylalanine, and tryptophan.
  - Transgenic crop plants contain a mutated **5-enolpyruvylshikimate-3-phosphate (EPSP) synthase gene**, which codes for an enzyme that is not inhibited by glyphosate, thus they exhibit **increased tolerance** to glyphosate, thereby increasing crop yield.
  - EPSP synthase is not found in higher animals, thus there are no harmful effects on humans consuming these crop plants sprayed with glyphosate.

- **Transgenic Atlantic salmon**
  - In a 1988 experiment, injection was initiated in Atlantic salmon eggs using a transgene consisting of an **eel pout's anti-freeze protein gene promoter** fused to the **growth hormone cDNA from Chinook salmon**. The anti-freeze protein gene promoter is active when the salmon is bred in the cold climates, thus the growth hormone is actively expressed.
  - It was discovered that transgenic fish grow up to ten times faster than non-transgenic fish, thus improving yield.

- **Virus-resistant animals**

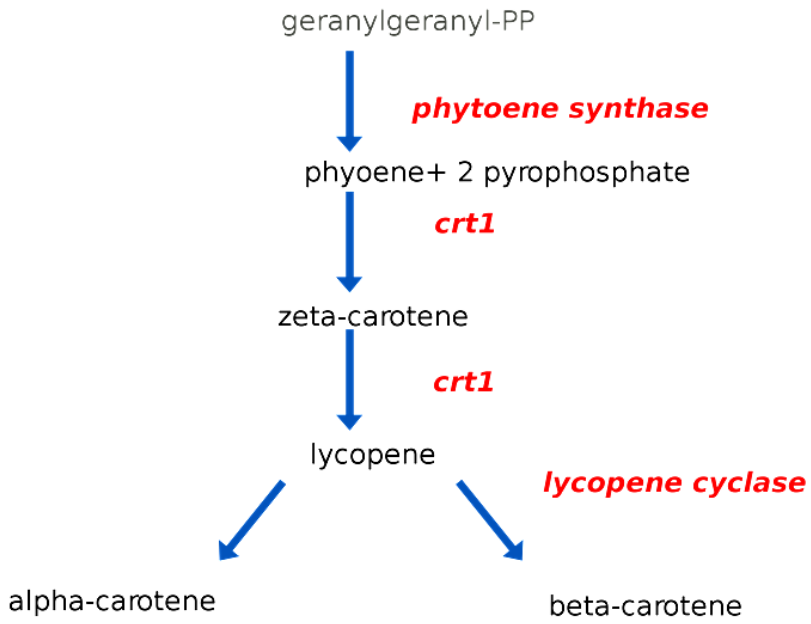
  Transgenic animals are also being produced for resistance to viral infections. **Avian leukosis virus (ALV)** is a major viral pathogen of chickens. The availability of an ALV-resistant species of chickens would be of major commercial value.

  - Transgenic chickens have been produced to carry a **defective ALV genome**.
  - These chickens **produce viral RNA and envelope proteins,** but **not assembled** into viruses.
  - The synthesis of **large amounts of the retroviral envelope protein blocks the reproductive cycle of intact, pathogenic ALV**.
  - The ALV resistance can be inherited by several generations of chickens, indicating that the trait is stable.

(ii) Improving health in humans

Some commercial claims of how genetic engineering of plants and animals improves health in humans include:

- **Improving nutritional value of crops (E.g. Golden Rice)**
  - "Golden" rice is a transgenic plant that produces yellow rice grains containing **beta-carotene,** which is a **provitamin that our body converts to vitamin A.** This could help **prevent vitamin A deficiency** in half of the world's population that depends on rice as a staple food. Vitamin A deficiency leads to vision impairment and increases susceptibility to other diseases.
  - Golden rice was **designed to produce beta-carotene** (a precursor of Vitamin A) **in the endosperm** (i.e. the part of rice that people eat).
  - The rice plant can naturally produce beta-carotene which is a carotenoid pigment found in leaves. However the plant does not normally produce the pigment in the endosperm.
  - Golden rice was created by transforming rice with two beta-carotene biosynthesis genes.
    - ***psy*, phytoene synthase** from daffodil (*Narcissus pseudonarcissus)*
    - ***crt1*** from the soil bacterium, *Ereinia uredovora*
  - The ***psy* and *crt1* genes** were **transformed into the rice nuclear genome** and **placed under the control of an endosperm specific promoter** so that they are only expressed in the endosperm.
  - lyc, lycopene cyclase gene that is naturally found in the wild type rice **process the lycopene to beta-carotene in the endosperm**, giving the rice the distinctive yellow colour for which it is named.



A simplified overview of the carotenoid biosynthesis pathway in Golden Rice

- **Production of pharmaceuticals**
  - Example 1: Hepatitis B vaccines have been successfully produced by transgenic plants and are currently undergoing clinical trials. Hepatitis virus infects liver cells and cause liver failure in humans.
  - Example 2: An antibody that interferes with the bacteria that cause tooth decay has been successfully produced by transgenic tobacco plants and is also currently undergoing clinical trials.
  - Example 3: Transgenic animals can potentially be used to produce and secrete valuable proteins in their milk. In a trial experiment, transgenic sheep were produced that secrete either of two human proteins, the blood-clotting protein factor IX or the elastase inhibitor α1-antitrypsin, in their milk. The transgenic sheep exhibited no apparent side effects from the production of either human protein in their milk. Nevertheless, further research must be done before the potential applicability of this approach can be adequately evaluated.

(iii) Improving quality of produce

- **Extension of shelf life of produce**

  Certain fruits like tomatoes tend to ripen too quickly. They become soft before they reach the marketplace. Therefore, large amounts of these fruits may be spoilt en route to their destination and this can result in massive losses commercially. Shelf-life of tomatoes can be extended using the **antisense RNA approach**.

  - The first genetically engineered plant product approved for human consumption was the **FlavrSavr™ tomato**.
  - **Polygalacturonase** is one of the many genes **expressed during ripening of tomatoes and results in softening of the hard flesh**.
  - Transgenic plants contain an **antisense polygalacturonase mRNA**. This results in the formation of **mRNA-antisense RNA hybrid**, which **prevents the polygalacturonase mRNA from being translated** and the enzyme will not be synthesized.
  - It ensures tomatoes are not too ripe and soft when it reaches the consumer in the market place.

- **Production of useful biochemicals**

  Transgenic plants with high expression of biochemical produce will reap commercial benefits.

  - **Laurate**, a fatty acid produced by **canola**, has been used extensively in the soap industry. Genetically modified canola has been inserted with the gene responsible for laurate production, resulting in the production of oil containing about 40% laurate.
  - Transgenic canola is able to produce a much higher percentage of laurate as compared to the wild-type species.

- **Improving muscle:fat ratio in animals**

  Research is currently ongoing to produce transgenic animals with leaner meat.

  - Transgenic pigs possess **extra copies of growth hormone genes** resulting in enhanced growth hormone levels which might result in leaner pigs with improved meat quality and with faster growth.
  - Results have shown that these pigs do grow at a faster rate under a protein diet and were leaner.
  - However, they exhibited several undesirable side effects, such as sterility and lethargy. Further research is necessary to curb these side effects.

(b) Social, Environmental and Ethical Issues of Genetically Modified Organisms

As the technology of genetic engineering advances, there is concern about the possible hazards of genetically modified organisms. Like all new technology, GMOs pose some risk, both known and unknown. The following are some concerns:

(i) Social Issues

1) Transfer of antibiotic resistance markers

While there is no evidence to show that any of the transgenes found in genetically modified foods are injurious to humans, opponents of genetic engineering and GMOs have focused on the **potential threat** to human health. It is questioned whether the plasmid vectors on which antibiotic resistance genes are inserted as markers, may be **accidentally transferred** from transgenic plants to pathogenic microorganisms which may result in increase in resistance to clinically important antibiotics.

2) Probability of introducing novel allergens

GM food might cause allergies. People with allergies could suffer reactions after unwittingly consuming genetically modified foods containing proteins introduced from sources they are allergic to. Some consumers are worried that introduced gene, toxic against pests could also cause the plant to alter the pollen of the plant, thereby affecting the health of humans already prone to pollen allergies.

3) Possibility of GM food being toxic or carcinogenic

Many consumers are wary of GM food, fearing that introduced genes could prove harmful to human health. E.g. if transgenes can cause a plant to produce toxins at higher levels than are present naturally, they may be toxic or carcinogenic when consumed on a long term basis.

4) Monopolistic behaviour of biotechnology companies

Seed companies have traditionally sought to control their product by limiting farmers from saving seeds for future planting. In 1998, a U.S. patent was granted for producing plants with sterile seed called the "technology protection system". The **terminator gene** is likely to be bred into many GMO seeds, causing second generation seeds to be sterile and farmers have no choice but to purchase a new batch of GMO seeds. Country farmers will become dependent on biotechnology companies and may be driven further into poverty without resources to purchase new seed each year.

Scientists have raised concerns about innovations in research that are not shared. Companies charge steep prices for the GMO seeds produced to recoup their investment. It raised fears that world food production may be dominated by a few large biotechnology companies.

5) Increasing dependence of developing nations on industrialized nations

The potential of using GMO for better yields and enhanced nutritional value can possibly alleviate problems of food shortage and malnutrition in developing nations. However in developing nations, farmers have agricultural practices of saving seeds from one harvest for the next. The increase in dependence for GMO seeds on large biotechnology companies highlights a need to ensure that developments in GM technologies do not benefit rich countries at the expense of the poor.

6) Impact on international trade

Europe has been much more hesitant than the United States in accepting GM products in processed food. European Union regulations require labelling of GM food products because of their strong belief that consumers have a right to know how their food is produced. The response of European nations and individual governments towards US GM food has become a major trade issue between the United States and these countries.

(ii) Environmental issues

1) Reduction in biodiversity

Genetically modified Bt crops may **alter ecology** of natural plant and insect populations. It is feared that **insects may quickly become resistant** to Bt in GM crops. On the other hand, some scientists worry that Bt would will kill non-target insects and affect the food chain and population of organisms.

The highly publicized Cornell University monarch butterfly study showed a high death rate after monarch larvae were fed transgenic Bt corn pollen. Subsequently, Iowa State University cautioned that findings obtained by the Cornell study were done under laboratory conditions and may not reflect actual field conditions. Efforts are underway to evaluate this danger in the field.

2) Transfer of transgenes to related organisms

Scientists have shown that genetically modified rapeseed (canola) pollen was spread to wild radish weed relatives in nearby fields. It demonstrated that new strains of weeds resistant to herbicides may be produced. Herbicide-resistant genes from GM plants may transfer to other plants through **cross pollination** to form superweeds. Superweeds would severely decrease crop productivity.

(iii) Ethical issues

1) Tampering with nature

The ethical issues of genetic modification for food production appear to be based mainly on religious grounds. Many objected that genetically modifying organisms will mean tampering with nature and hence going against the natural way of life (avoid the phrase 'playing God').

2) Lack of mandatory food labelling in some countries

Different countries have different laws in requiring the labelling of GM food. In US, there is no mandatory labelling of GM food or GM processed food. Consumers are advocating mandatory labelling of all GM foods based on consumers' right to know and choose.

Religious groups are concerned that GM foods might contain genes from animals prohibited by their religion. The Jewish and Muslim communities have not yet resolved whether GM food containing a gene derived from swine is acceptable. Therefore proper labelling may make compliance with religious beliefs easier.

4. Stem Cells

Stem cells are **unspecialized cells that can divide, during a single division, into one identical daughter cell and one more specialised daughter cell, which can undergo further differentiation**. When unspescialized stem cells divide and develop to specialized cells, the process is called **cell differentiation**. Stem cells are common to all multi-cellular organisms.

(a) Features of Stem Cells

Stem cells differ from other kinds of cells in the body. All stem cells, regardless of their source, have three general features:

(i) They are **unspecialized**.

A stem cell does not have any tissue-specific structures that allow it to perform specialized functions. However, unspecialized stem cells can give rise to specialized cells like heart muscle cells, blood cells, or nerve cells which play specific roles.

(ii) They are **capable of dividing and renewing themselves for long periods**.

Specialised cells like muscle cells, blood cells, or nerve cells do not normally replicate themselves. Stem cells may divide many times. Of the two daughter cells formed by mitosis of a stem cell, one cell remains unspecialised while the other differentiates.

(iii) They can **give rise to specialized cell types**.

Scientists are just beginning to understand the signals inside and outside cells that trigger stem cell differentiation. The **internal signals are controlled by a cell's genes** while the **external signals for cell differentiation include chemicals secreted by other cells**, **physical contact with neighbouring cells**, and **certain molecules in the microenvironment**.

Potency specifies **the stem cell's potential to differentiate into different cell types**:

- **Totipotent** stem cells
  - **Zygotic stem cells** (produced from the fusion of an egg and sperm cell) are totipotent.
  - These cells **can differentiate into any cell types to form whole organisms, and so are also pluripotent and multipotent**.
- **Pluripotent** stem cells
  - **Pluripotent** stem cells are the descendants of totipotent cells.
  - **Embryonic stem cells** from the blastocyst (a hollow ball-shaped mass of cell formed a week after fertilisation) are pluripotent.
  - These cells **can differentiate into almost any cell type to form any organ or type of cell and so are not totipotent but are multipotent**.
- **Multipotent** stem cells
  - **Blood / hematopoietic stem cells** (found in the bone marrow) are multipotent.
  - These cells can **differentiate into a limited range of cell types, usually of a closely related family of cells** and so are not pluripotent or totipotent.
  - **Blood / hematopoietic stem cells** can only **differentiate into red blood cells, white blood cells, platelets, etc.**
  - Another example is umbilical cord stem cells, which are also multipotent.
- Unipotent stem cells can differentiate into only one cell type, but have the ability of self-renewal which distinguishes them from non-stem cells, e.g. liver cells.

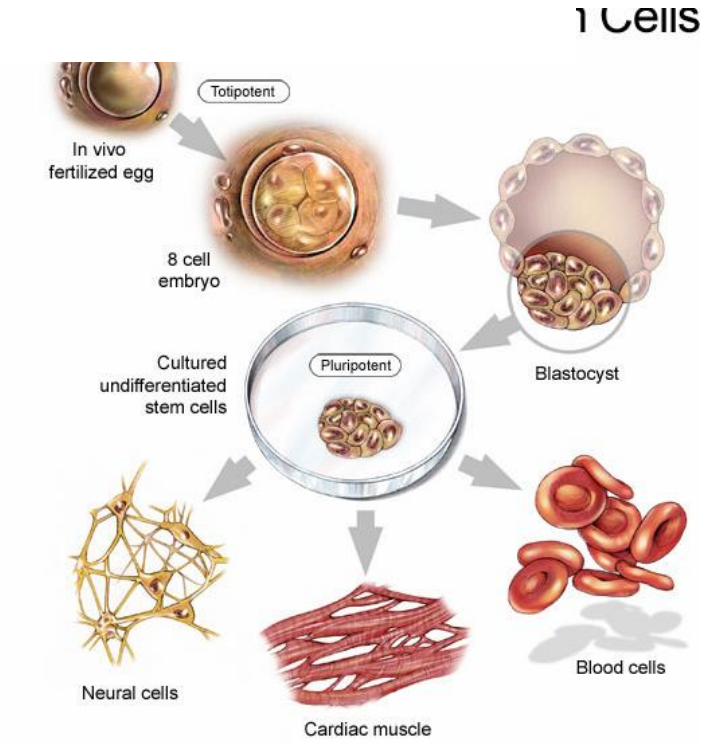


Diagram showing pluripotency of embryonic stem cells

(b) Functions of Stem Cells in Living Organisms

(i) Embryonic stem (ES) cells

- ES cells are obtained from a blastocyst. A blastocyst is an embryonic stage in mammals – approximately 5 – 7 days old in humans and consisting of 50 – 150 cells.
- **ES cells obtained from the blastocyst are pluripotent**, giving rise to all derivatives of the three primary germ layers: **ectoderm, endoderm and mesoderm** during development.
- These germ layers subsequently **give rise to the multiple specialized cell types** that make up the heart, lung, skin, and other tissue.

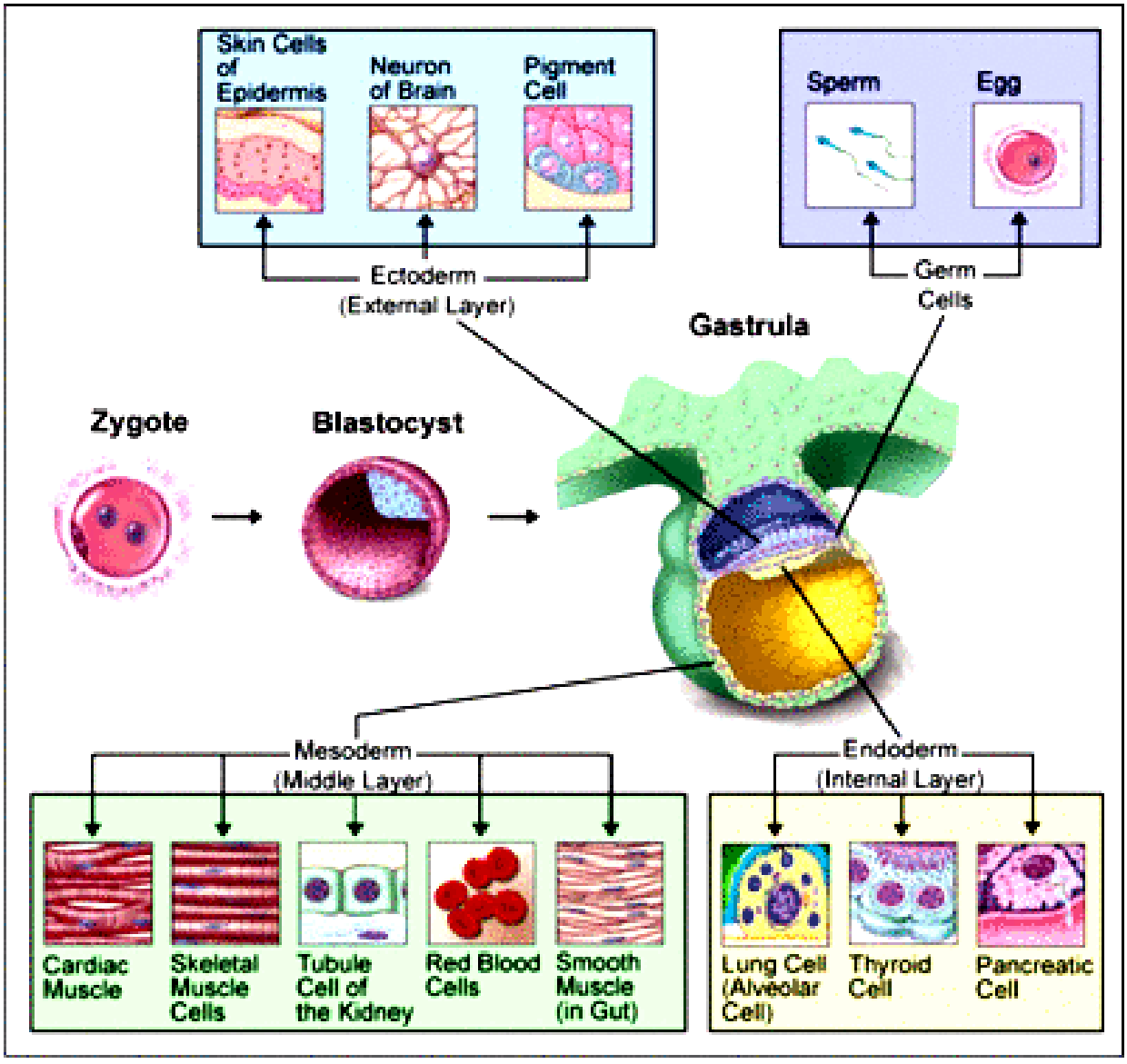


Diagram showing the fate of cells in a developing embryo

(Adapted from http://content.answers.com/main/content/wp/en/thumb/9/94/400px-Cell_differentiation.gif)

(ii) Blood / hematopoietic stem cells

- Adult stem cells are rare and few in numbers but have been identified in many organs and tissues. Adult stem cells are reported to be found in brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin and liver.
- Their primary function is to **maintain the steady state functioning of a cell by generating replacements for cells lost through disease, tissue injury or normal wear-and-tear.**
- Adult stem cells typically generate the cell types of the tissue in which they reside. A blood stem cell in the bone marrow, for example, normally gives rise to the many types of blood cells such as red blood cells, white blood cells and platelets.

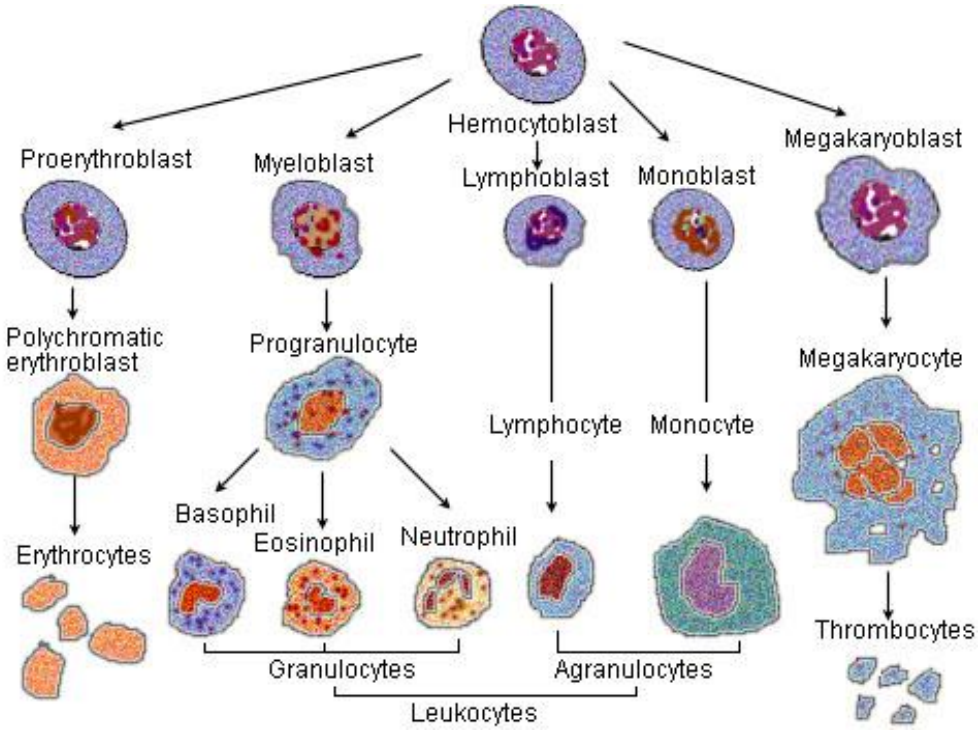


Diagram showing differentiation of blood stem cell (hemocytoblast)

5. Gene Therapy

Of the over 4000 inherited human diseases catalogued to date, only a few are currently treatable. For many of these diseases, the missing or defective gene product **cannot be supplied exogenously**, like how insulin can be supplied to diabetics. There are three main reasons for this:

- Most proteins / enzymes are **unstable** and **degrade before reaching their sites of action** in the body.
- Even if the enzymes are successfully delivered to their sites of action in the body, their **enzymatic activity may not persist for long term**.
- **Cell surface membranes are impermeable to large macromolecules**, thus enzymes must be synthesized in the cells where they are needed.

Gene therapy involves the transfer of a normal (wild-type) copy of a gene to the genome, which carries defective copies of the gene, to correct a genetic defect. Gene therapy offers a promising approach to successful treatment of genetic diseases. If gene therapy is successful, the transgene will synthesize the missing gene product and restore the normal phenotype, thus eliminating disease.

(a) Techniques

There are two types of gene therapy.

(i) Somatic cell or **non-heritable gene therapy**:

- Transgene is inserted into somatic cells.
- It can be used to treat symptoms of the individual but will not cure the disease.
- Defective gene is still present after therapy andmay still be transmitted to future generations.

(ii) Germ cell or heritable gene therapy:

- Transgene is inserted into reproductive / germ cells.
- Transgene may be transmitted to future generations such that they will not suffer from disease.
- However, this is still not performed on humans due to ethical issues.

In most gene therapy procedures, the therapeutic gene is inserted into the target cell by a **vector**. There are two delivery systems that are used to insert transgene into human cells:

- **Viral delivery system**:
  - Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner.
  - Genetically altered viruses are the most common vector to carry the transgene to target cells.
    - **Attenuated viruses** are **viruses that have their disease-causing genes removed**. Therapeutic genes are inserted and these viruses are used to infect target cells such as the patient's liver or lung cells.
    - The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state.
  - Different types of viruses used as gene therapy vectors include :-
    - Retroviruses – RNA viruses that can generate double-stranded DNA copies of their RNA genomes. These DNA copies of its genome and the therapeutic genes become integrated into the chromosomes of host cells. However, **integration of the therapeutic gene in the human genome is random**.
    - Adenoviruses – Non-enveloped, double-stranded DNA viruses that infect respiratory / intestinal cells in humans.
    - Adeno-associated viruses (AAV) - Small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19. However, due to the small nature of AAV, large genes cannot be carried by AAV.
    - Herpes simplex viruses - Double-stranded DNA viruses that can infect neurons.

- **Non-viral delivery systems**:

  Besides virus-mediated gene-delivery systems, there are several possible non-viral options for gene delivery.

  - **Microinjection**
    - This is the simplest method as **therapeutic gene is directly introduced into target cells**.
    - This approach is limited in its application because it can be **used only with certain tissues** and **requires a large amount of therapeutic genes**.

- **Liposome-mediated delivery**
  - Another non-viral approach involves the **creation of an artificial phospholipid sphere with an aqueous core** known as **liposome**.
  - The liposome carries the therapeutic DNA in the aqueous core and is **capable of passing the therapeutic gene through the target cell's membrane**.

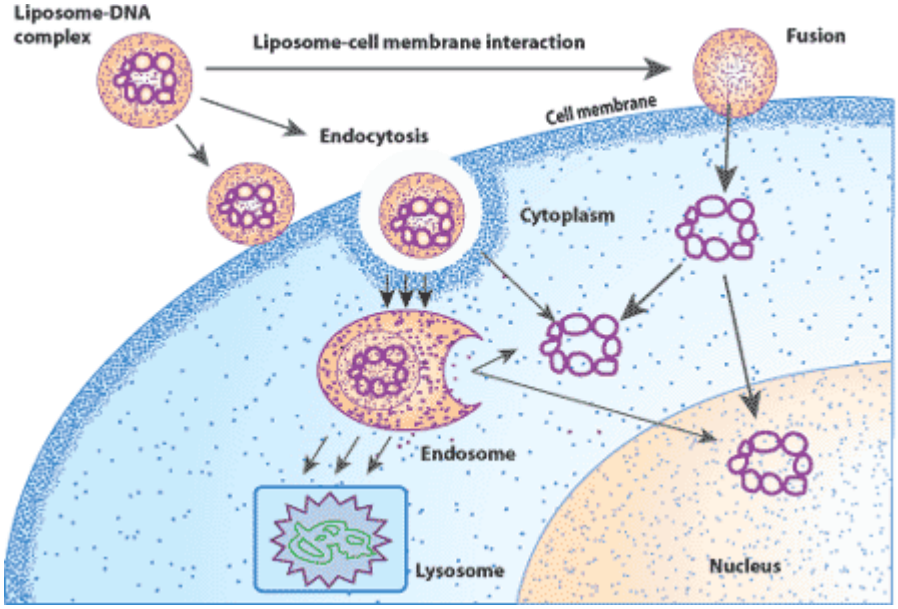


Diagram showing liposome-mediated delivery of genes

(Taken from http://www.vaxim.com/img_mechanisms.gif)

- **Linking therapeutic DNA to signal molecules**
  - Therapeutic DNA also can enter target cells by **chemically linking the DNA to a molecule that will bind to target cell's specific receptors**.
  - Once bound to these receptors, the therapeutic DNA constructs are enters the target cell via endocytosis.
  - This delivery system tends to be less effective than other options.

- **Introduction of 47th chromosome**
  - Researchers also are still experimenting with introducing a 47th (artificial human) chromosome into target cells. This chromosome would **exist autonomously alongside the standard 46 chromosomes** and will not affect their functions or cause any mutations.
  - It would **be a large vector capable of carrying substantial amounts of genetic information**.
  - Due to its construction and autonomy, it **would not trigger a response from the body's immune system**.
  - A problem with this potential method is the **difficulty in delivering such a large molecule to the nucleus of a target cell**.

(b) Importance and Benefits in the treatment of SCID & CF

(i) Treatment of <u>Severe Combined Immunodeficiency (SCID)</u>

SCID is a genetic disorder in which the **immune system is crippled** and patients are extremely vulnerable to infectious diseases.

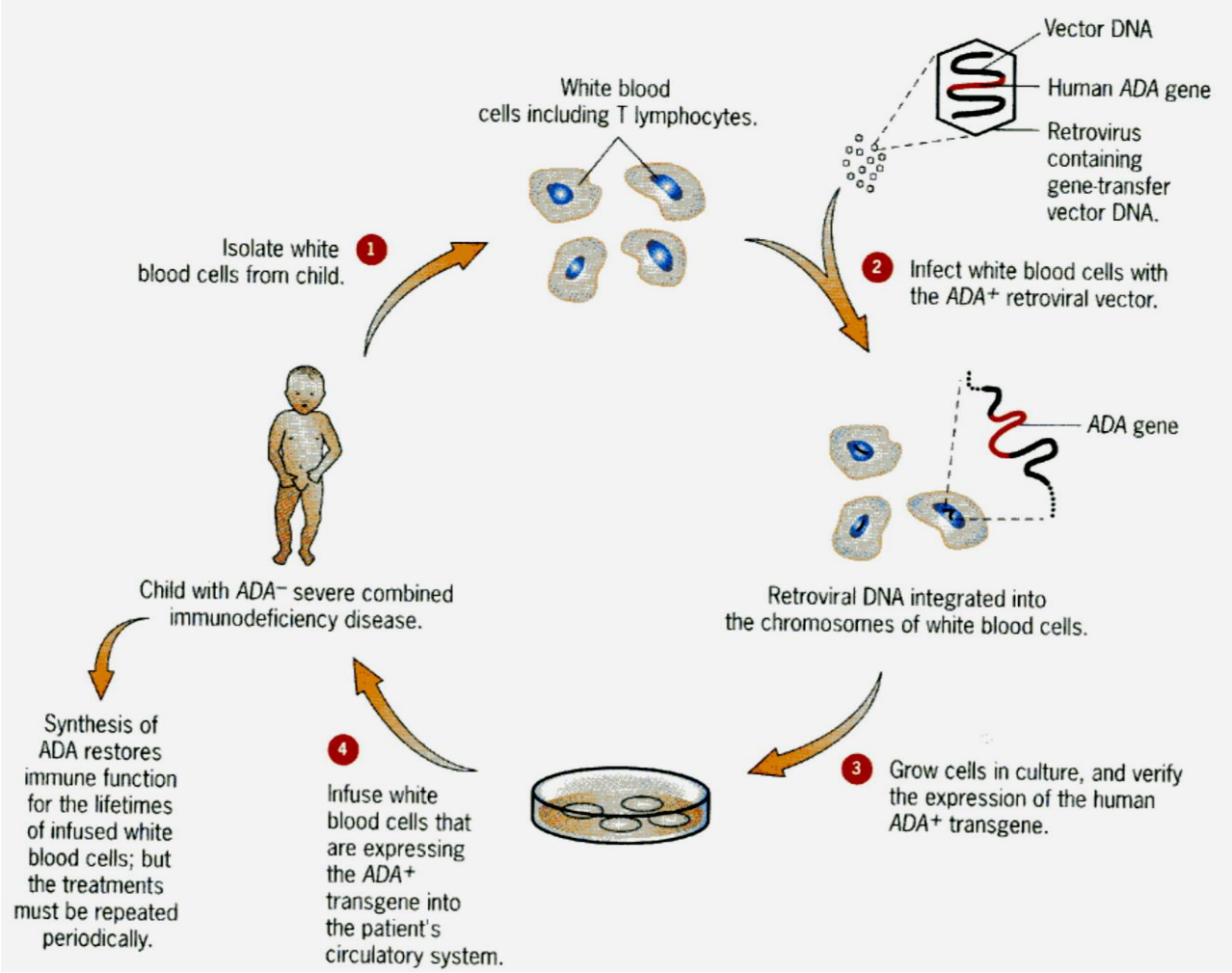
- Due to the **<u>low levels / absence of T-lymphocytes and B-lymphocytes</u>, antibodies are not produced against foreign antigens** (from bacteria or viruses).
- Patients are **susceptible to opportunistic infections** and suffer chronic infections such as ear infections, oral yeast infections, chronic diarrhea, pneumonia, measles and **usually die within their first two years of life**.
- **Fatality is higher in children** when the condition is left untreated.

There are two main forms of SCID, which is caused by mutation of different genes:

- **<u>X-linked SCID</u>** is the most common form, **affecting more males than females**.
  - It is due to **mutation of interleukin-2 (IL2) receptor gamma gene** that **encodes the gamma chain of interleukin receptors.** This gene is found on the **X chromosome.**
  - Without functional interleukin receptors, **immune cells cannot be stimulated to develop and differentiate into T and B lymphocytes**.
- **Adenosine deaminase (ADA) deficient SCID** is due to a **deficiency in ADA**.
  - ADA is necessary for the **breakdown of purines**.
  - The **lack of ADA causes accumulation of dATP**, which will **inhibit the activity of ribonucleotide diphosphate reductase**, the enzyme that **reduces ribonucleotides to generate deoxyribonucleotides** (dNTP).
  - The effectiveness of the immune system depends upon lymphocyte proliferation and hence dNTP synthesis. Without functional ribonucleotide reductase, **lymphocyte proliferation is inhibited** and the **immune system is compromised**.

While it has been proven over several generations now that bone marrow transplants can save the lives of SCID children, transplants do not always work or do not always completely correct the defects. Therefore gene therapy has been used in the treatment of SCID.

- The first gene therapy trials were performed in 1990, with peripheral T-lymphocytes.
  - **T-lymphocytes** were extracted from the patients suffering from ADA-deficient SCID.
  - Therapeutic DNA was inserted in these cells using a **retroviral vector**.
  - The cells are **grown in a culture medium** and **verified for expression of ADA transgene**.
  - The genetically modified cells were then transferred back to the patients.
- In 2000, the first gene therapy success resulted in SCID patients with a functional immune system. However, the success of these early trials is debated.
  - Periodic tests on the patients show that their re-engineered cells are surviving and producing the ADA enzyme. However the **lifespan of these cells are short**, therefore these patients have to **continue to receive gene therapy**.
  - These trials were stopped when it was discovered that two of ten patients in one trial had developed leukemia resulting from the insertion of the gene-carrying retrovirus near an oncogene. In 2007, four of the ten patients have developed leukemias.
  - Currently, transduction of the missing gene to **hematopoietic stem cells** using viral vectors is being tested in ADA SCID and X-linked SCID.



Treatment of SCID using gene therapy

(Adapted from Snustad, Simmons and Jenkins *'Principles of Genetics'*)

(ii) Treatment of Cystic Fibrosis

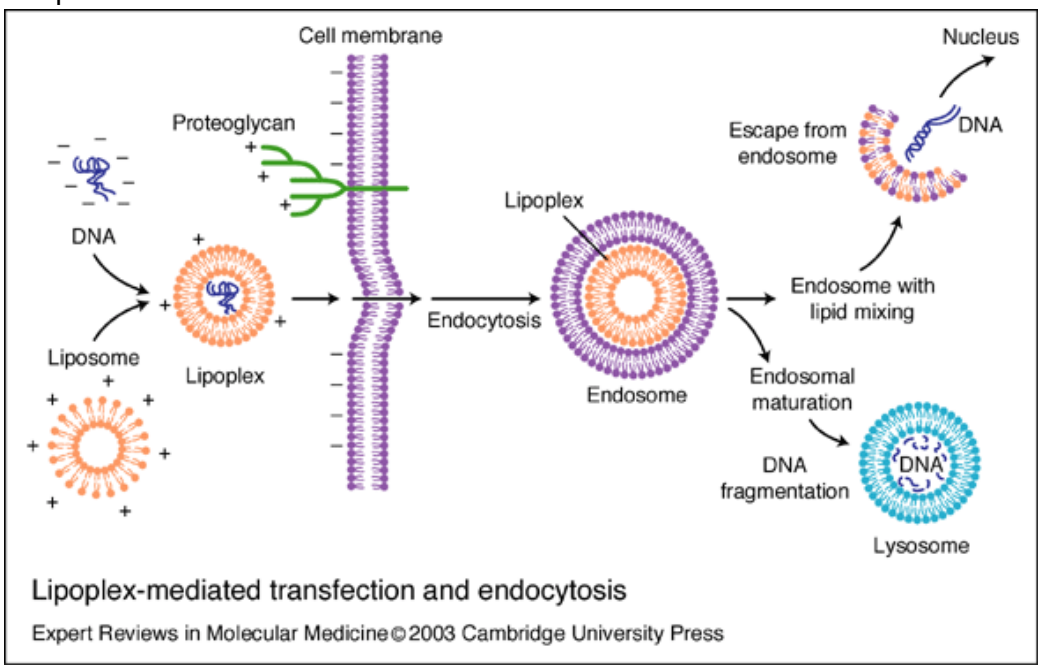
Cystic fibrosis is one of the most common inherited diseases in humans. It is inherited as an **autosomal recessive** mutation. Sufferers often have **reduced lifespan** due to the symptoms.

Cystic fibrosis is due to **mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene**, which codes for **CFTR protein**.

- Most cystic fibrosis patients have a mutated CFTR gene on **chromosome 7,** consisting of **deletion of three consecutive nucleotides**, resulting in **deletion of phenylalanine** from CFTR protein.
- In some other cases, the whole CFTR is missing from the membranes of these cells.
- CFTR protein function as **chloride ion channels** embedded in membranes of cells that line the respiratory tract, pancreas, sweat glands, intestine and other organs. It regulates the **transport of chloride ions out of these cells.**
- Mutated CFTR gene encodes a **non-functional CFTR protein**, resulting in **accumulation of chloride ions in cells**, causing **build up of thick sticky mucus on the surfaces of these cells**. This results in the symptoms associated with this disease:
  - **Excessively salty sweat**, a largely benign effect of the mutant gene due to chloride ions being trapped in the cells. This draws in positive charged ions, like sodium and form the salt, sodium chloride which is lost in the sweat.
  - Respiratory problems as lungs are clogged with thick mucus, which results in **interference with gaseous exchange**. Patients are often breathless due to inefficient gaseous exchange.
  - Mucus in lungs also tend to **trap bacteria leading to chronic lung infections** e.g. pneumonia.
  - Thick mucus often results in blocked pancreatic ducts and **reduced secretion of pancreatic enzymes needed for proper digestion**.
  - Building up of mucus in the digestive tract **interferes with absorption of digested nutrients** causing individuals to be malnourished no matter how much they eat.
  - **Males may be sterile and females have reduced fertility**. Men make normal sperm but are missing the vas deferens whereas females have fertility difficulties due to thickened cervical mucus (sperms cannot penetrate) and malnutrition disrupting ovulation and causing amenorrhea, the absence of a menstrual period in a woman of reproductive age.
  - Patients often **develop diabetes** although the cause is still relatively unknown.

In recent years, cystic fibrosis can be successfully treated by gene therapy.

- The normal CFTR gene can be introduced to **lung epithelial cells**. Since cystic fibrosis is an autosomal recessive disease, only one copy of the normal gene is required.
- Adenoviral delivery system is used since adenoviruses infect lung cells. These genetically modified adenoviruses are introduced into patients via aerosol sprays. However, it was not very successful as the levels of CFTR gene activity were too low.
- Therefore **liposome-mediated delivery system** is also used in the gene therapy of cystic fibrosis. Liposome-mediated delivery system is used where cationic liposome complexed with a complementary DNA encoding the CF transmembrane conductance regulator (CFTR) is introduced into patients' nasal epithelium.



Lipoplex-mediated transfection and endocytosis

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- Recent studies have shown **lipoplexes** to be useful in transfecting respiratory epithelial cells, so they may be used for treatment of genetic respiratory disease such as cystic fibrosis.
- To improve the delivery of therapeutic DNA into the cell, the DNA must be protected from damage and its entry into the cell must be facilitated.
- This involves the creation of an artificial phospholipid sphere with an aqueous core known as **liposome**. The **therapeutic DNA can be covered with liposome and it is called a lipoplex**.
- The lipoplex is capable of passing the therapeutic gene through the target cell's membrane.
- Cationic lipids, due to their positive charge, naturally complex with the negatively charge DNA. As a result of their charge they interact with the cell membrane, endocytosis of the lipoplex occurs and DNA is released into the cytoplasm.
- The cationic lipids also protect against degradation of the DNA by the cell.

(c) Limitations of Gene Therapy

There are several factors that keep gene therapy from becoming an effective treatment for genetic diseases:

- **Short-lived nature of gene therapy**
  - Before gene therapy can become a permanent cure for any condition, the therapeutic gene introduced into target cells must remain functional and the cells containing the therapeutic gene must be long-lived and stable.
  - Problems with integrating therapeutic DNA into the genome and short lifespan of these genetically modified cells prevent gene therapy from achieving any long-term benefits. Patients still have to undergo multiple rounds of gene therapy.
  - E.g. Cystic fibrosis patients have to undergo repeated rounds of gene therapy.
- **Incorrect insertion of therapeutic gene** resulting in other diseases
  - One of the problems of gene therapy using retroviruses is that the DNA of the virus is **usually inserted randomly** in the genome of the host. This could dirupt the normal functioning of genes regulating cell division, resulting in **uncontrolled cell division, causing cancer**.
  - E.g. Two patients treated for SCID developed a form of T-cell proliferation similar to leukaemia. The therapeutic DNA was inserted next to a gene coding for a specific leukaemia inhibitor. As such, most SCID-related gene therapy trials were stopped worldwide.
- **Triggering of immune response resulting in rejection**
  - When a foreign vector is introduced into human tissues, the immune system elicits a response to counter the vector. Therefore, there is always the **risk of stimulating the immune system when a foreign vector is introduced** and this reduces effectiveness of gene therapy.
  - Furthermore, the immune system is able to **mount an enhanced response to the same foreign vectors** which it has encountered before and this makes it **difficult for gene therapy to be repeated** in patients.
- **Problems with finding suitable viral vectors**
  - Viruses, being a pathogen, may exhibit **unknown toxicity**, and **elicit immune and inflammatory responses**. They could also affect control of gene expression in target cells. In addition, there is always the fear that the viral vector, once inside the patient, **may recover its ability to cause disease**.

- **Inability to treat multi-gene disorders**
  - Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are **caused by the combined effects of many genes**. Multi-gene disorders such as these would be especially difficult to treat effectively using gene therapy.
- **Inability to target multiple diseased sites**
  - In diseases which **affect more than one organ or tissue**, gene therapy may only be able to target one specific type of tissue.
  - E.g. in gene therapy treatment of cystic fibrosis, treatment is usually administered by aerosols containing viral vectors with transgene. The target cells are the epithelium of the respiratory system. Other diseased sites, such as the pancreas is not treated.

(d) Social and Ethical Issues

Some of the ethical and social considerations for the use of gene therapy in the treatment of diseases are as follows:

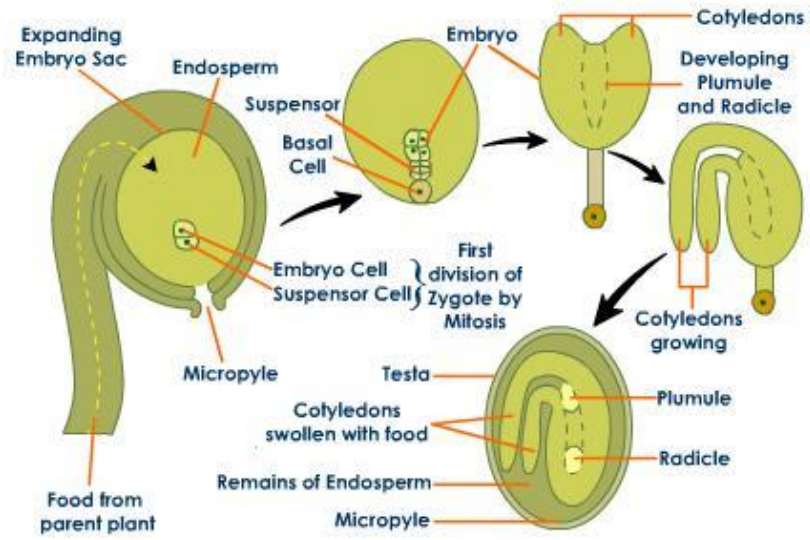
- **Possibility of genetic discrimination by society**
  - What is normal and what is a disability or disorder, and who decides?
  - Are disabilities diseases? Do they need to be cured or prevented?
  - Does searching for a cure demean the lives of individuals presently affected by disabilities?
- **Discrimination of the poor**
  - Preliminary attempts at gene therapy are exorbitantly expensive. Who will pay for their use?
  - Will the poor have access to such technology considering their inability to pay for such treatments? How would this affect social stratification?
- **Tampering with nature**
  - Religious groups may view gene therapy as going against the natural way of life.
  - Is somatic gene therapy more or less ethical than germ-line gene therapy?

APPENDIX

Other Tissue Culture Techniques (Optional; Good to know)

(a) Source of Explant

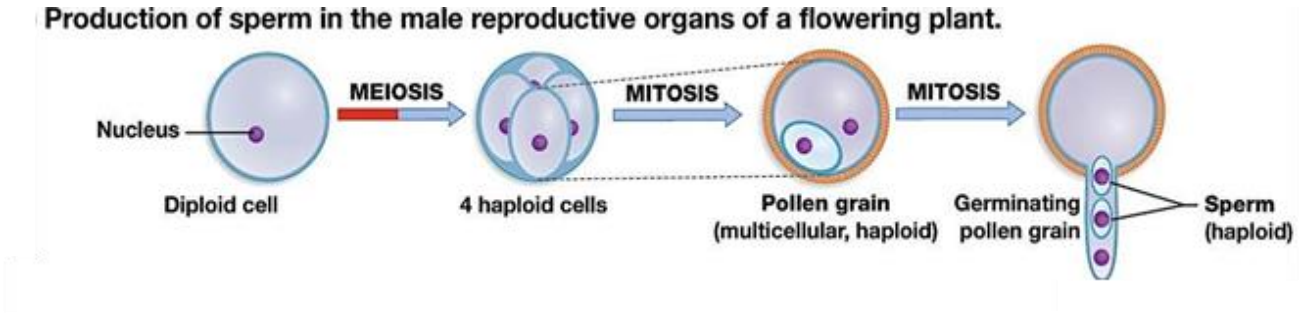
- **Embryo culture**
  - Embryos from fertilised zygote used as explant to initiate callus cultures.
  - Useful for overcoming dormancy of seeds. The culture of mature embryos from ripened seeds is used to eliminate seed germination inhibitors or to shorten the breeding cycle.
  - Useful for embryo rescue. Crosses between incompatible varieties of plants will result in post-zygotic incompatibility, whereby a zygote is produced but not accepted by the endosperm. The embryo will disintegrate due to the lack of nutrition from endosperm. Therefore hybrid seedlings are not produced. Its potential to resume normal growth may be realized if supplied with the proper growth substances. This will induce continued embryogenic growth and seedling formation.



Normal Development of embryo after fertilisation

- **Anther culture**
  - Explant: pollen grains (within anthers)
  - Anther contains haploid microspores (immature pollen grains) which develop into pollen grains containing male gametophyte.
  - Instead of producing gametes, microspores become haploid plants by the induction of embryogenesis from repeated divisions of microspores.
  - Haploid microspores → Haploid embryos or callus tissue → Haploid plants.
  - Eliminates many cycles of selection and back-crossing required to produce homozygous plants.

- No masking of genes; all phenotypic effects can be observed (recessive traits).
  - Lethal genes eliminated since recessive alleles are expressed; cell dies.
- Haploid plants are sterile
  - The chromosome complement of these haploids can be doubled by colchicine treatment to yield fertile homozygous diploid plants.
  - Therefore production of plants with homozygous alleles for desired trait.



Normal male gametophyte development: Each of the four haploid microspores develops into a pollen grain. Within each pollen grain there are two sperms.

(b) Treatment of explant

- **Procedure for preparation of protoplast culture**

  Objective: Removal of cell walls without causing irreversible damage to the released protoplasts and the maintenance of a suitable osmotic environment to stabilize the protoplasts.

  - **Choosing of explant:**
    - Isolated from meristematic tissue.
    - Surface sterilised with sodium hypochlorite.
  - **Isolation of protoplasts:**
    - For leaf explants (e.g. leaf mesophyll), the epidermis of the leaf is peeled off and exposed to enzymes.
    - Cells are plasmolysed in mannitol, sorbitol or sucrose, so that the protoplast can contract away from the cell wall.
    - Enzymatic treatment to remove cell wall
      - Incubation with fungal pectinase and cellulase enzymes to digest the cell wall
      - Advantages: Large scale reproducible isolation of protoplasts from various tissues is possible. Osmotic shrinkage is minimum and the deleterious effects of excessive plasmolysis are minimized. Cells are intact and are not injured as in the case of mechanical methods of isolation.

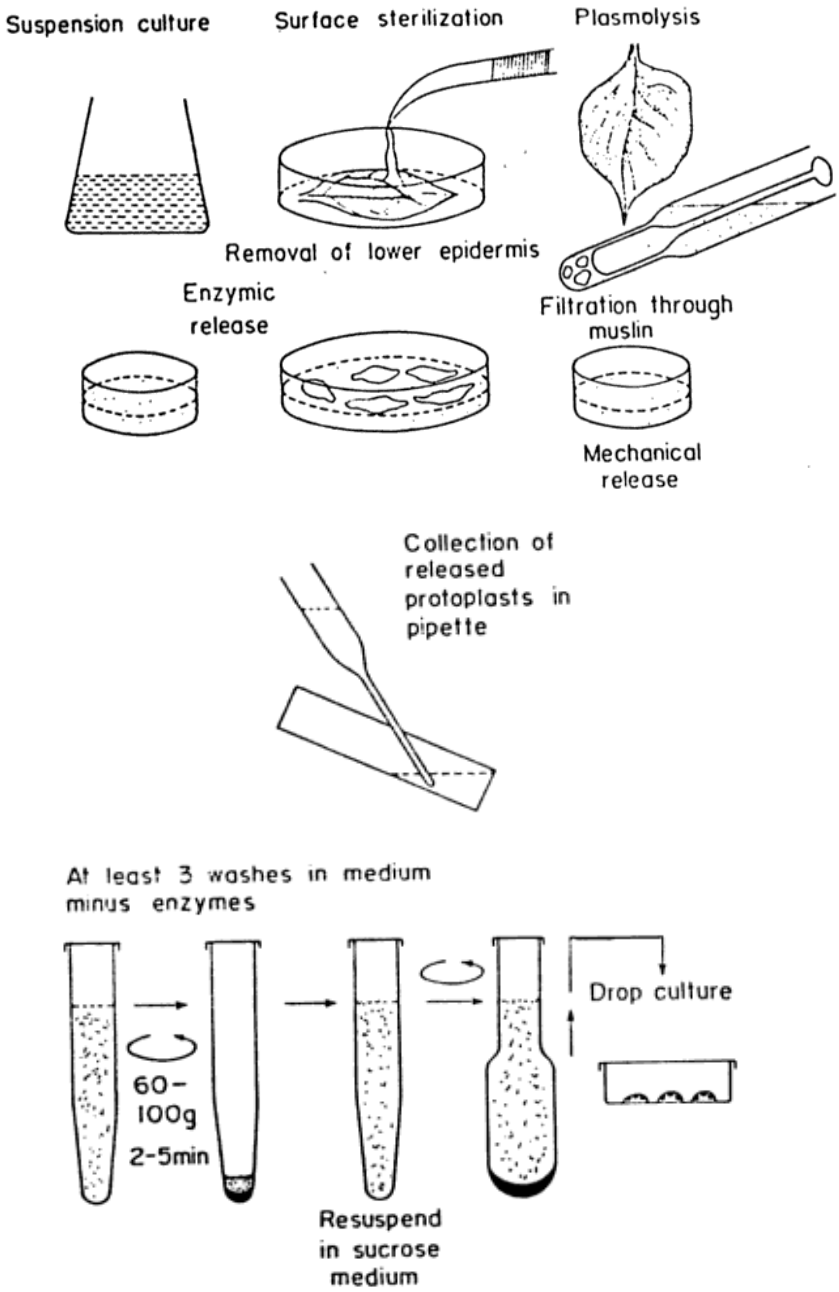
- After enzyme treatment, protoplast suspensions are collected by centrifugation, washed in enzyme-free medium and separated from cell and cell debris by sucrose floatation (sucrose gradient). It separates them based on their different specific gravity.
- Cell debris will pellet.
- Buoyant protoplast will float at sucrose surface.

➢ **Culture of protoplasts:**

- Protoplasts suspended in a suitable medium in order to allow them to reform a cell wall and initiate divisions.
- Important ingredients in protoplast culture medium: Osmotic stabilizers and plant growth substances
- Cell colonies formed from protoplasts can be transferred to fresh nutrient media for further growth and multiplication.

➢ **Protoplast fusion:**

- Protoplasts from different plants are induced to fuse by addition of chemicals such as polyethylene glycol (PEG) or calcium.
- Another method is via electro-fusion; applying short pulses of direct electrical current.
- Identification and selection of hybrid cells



Basic Biotechnology (2007) Rev Fr Dr S Ignacimuthu, s.j., Tata Mcgraw-Hill

(c) To create seeds

- **Somatic Embryogenesis**
  - Embryogenesis: The process of forming an embryo, usually from zygote (fusion of an egg (in ovule) and sperm cell (in pollen) during fertilisation). The zygote will undergo asymmetrical cell divisions to give rise to an embryo.
  - Somatic embryogenesis: Embryos derived from somatic cells.
  - Technique is useful for overcoming various environmental and genetic factors that prevents fertilization.
  - Plant growth regulators in tissue culture medium first induce callus formation, then changed to induce embryos from callus; Indirect embryogenesis (indirect formation of somatic embryos from callus)
  - Cultures should be grown in high level of Auxin, low level of Nitrogen for the induction of embryogenesis.
  - Artificial seeds prepared by encapsulating somatic embryos with nutrient-rich calcium alginate, which can be used as functional seeds for distribution and transport.
  - E.g. asparagus, celery, begonias, African violets.

Benefits of Stem Cell Research (Optional Reading)

There are many ways in which human stem cells can be used in basic research and in clinical research. However, there are many technical hurdles between the promise of stem cells and the realization of these uses, which will only be overcome by continued intensive stem cell research.

Studies of human embryonic stem cells may yield information about the complex events that occur during human development. A primary goal of this work is to identify how undifferentiated stem cells become differentiated. Scientists know that turning genes on and off is central to this process. Some of the most serious medical conditions, such as cancer and birth defects, are due to abnormal cell division and differentiation. A better understanding of the genetic and molecular controls of these processes may yield information about how such diseases arise and suggest new strategies for therapy. A significant hurdle to this use and most uses of stem cells is that scientists do not yet fully understand the signals that turn specific genes on and off to influence the differentiation of the stem cell.

Human stem cells could also be used to test new drugs. For example, new medications could be tested for safety on differentiated cells generated from human pluripotent cell lines. Other kinds of cell lines are already used in this way. Cancer cell lines, for example, are used to screen potential anti-tumour drugs. But, the availability of pluripotent stem cells would allow drug testing in a wider range of cell types. However, to screen drugs effectively, the conditions must be identical when comparing different drugs. Therefore, scientists will have to be able to precisely control the differentiation of stem cells into the specific cell type on which drugs will be tested. Current knowledge of the signals controlling differentiation fall short of being able to mimic these conditions precisely to consistently have identical differentiated cells for each drug being tested.

Perhaps the most important potential application of human stem cells is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including Parkinson's and Alzheimer's diseases, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis. In people who suffer from type I diabetes, the cells of the pancreas that normally produce insulin are destroyed by the patient's own immune system. New studies indicate that it may be possible to direct the differentiation of human embryonic stem cells in cell culture to form insulin-producing cells that eventually could be used in transplantation therapy for diabetics.

To be useful for transplant purposes, stem cells must be reproducibly made to:

- Proliferate extensively and generate sufficient quantities of tissue.
- Differentiate into the desired cell type(s).
- Survive in the recipient after transplant.
- Integrate into the surrounding tissue after transplant.
- Function appropriately for the duration of the recipient's life.
- Avoid harming the recipient in any way.