PLANT AND ANIMAL BIOTECHNOLOGY

Plant tissue culture is the in-vitro aseptic culture of cells, tissues, or whole plants under controlled nutritional and environmental conditions, often to produce clones of plants.

The technique primarily relies on plant cells' totipotency which is the capacity of a single cell to express the whole genome during cell division. The ability of cells to change their metabolism, growth, and development is just as significant and essential for the regeneration of the entire plant.

Plant tissue culture technology is being widely used for large-scale plant multiplication. In addition to being used in research, they are now essential for plant propagation, disease eradication, and the generation of secondary metabolites.

Plant Tissue Culture

Plant Tissue Culture History

Gottlieb Haberlandt, in 1902 tried to cultivate individual palisade cells from leaves in knop's salt solution supplemented with sucrose. The cells sustained for a month stored starch but ultimately did not divide. Despite his failure, he is considered the father of plant tissue culture since his experiment set the stage for developing tissue culture technology.

Plant Tissue Culture Conditions

The choice of medium is based on the types of plant species; explants are used for culture for optimal response. All the nutrients required for a plant's proper growth and development should be present in the plant tissue culture media. Macronutrients, micronutrients, vitamins, other organic ingredients, plant growth regulators, a carbon source, and in the case of a solid medium, a few gelling agents make up the majority of its composition. Similarly, hormone levels and culture variables like temperature, pH, light intensity, and humidity also play an important role in the success of tissue culture.

- The minerals consist of macronutrients such as nitrogen, potassium, phosphorus, calcium, magnesium, and sulfur, and micronutrients such as iron, manganese, zinc, boron, copper, molybdenum, and cobalt.
- Vitamins are necessary for the healthy growth of plant cultures. The vitamins like thiamine (vitamin B1), pyridoxine (B6), and nicotinic acid (niacin). Other vitamins such as biotin, folic acid, ascorbic acid (vitamin C), and vitamin E (tocopherol) are sometimes added to media formulations.
- Plants also require an external carbon source; sugar. The most commonly used carbon source is sucrose. Other sources used are glucose, maltose, and sorbitol.
- The pH of the culture medium remains vital as it influences the uptake of various components of the medium and regulates a wide range of biochemical reactions. Most media are adjusted to a pH of 5.2–5.8. A higher pH may be required for certain cultures.

Plant Tissue Culture Media

- The most popular medium for in vitro vegetative propagation of various plants is Murashige and Skoog medium (MS medium). For culturing, either a solid or liquid medium can be employed.

- McCown's woody plant medium (WPM) has been widely used for tree tissue culture.
- Knudson's medium is used for orchid tissue culture and fern tissue culture.

Plant Tissue Culture Growth regulators

- Plant growth regulators (PGRs) are crucial for determining the development of plant cells and tissues in a culture medium.
- The most commonly used plant growth regulators are auxins, cytokinins, and gibberellins.
- The high auxin concentration often favors the development of roots. The most commonly used auxins are IAA (indoleacetic acid), IBA (indolebutyric acid), NAA (naphthaleneacetic acid), and 2,4-D (2,4 dichlorophenoxyacetic acid).
- Cytokinins promote cell division and shoot growth. The most commonly used cytokinins are BAP (benzylaminopurine), zeatin, Isopentenyl adenine (2-ip), and kinetin. Cytokinins are generally dissolved in dilute HCl or NaOH.
- A clump of undifferentiated cells called a callus develops when auxin and cytokinin levels are balanced.

Plant Tissue Culture Vessels

- Another critical aspect in plant tissue cultures is the management of the gaseous hormone ethylene. In closed culture vessels used for in vitro plant growth, ethylene builds up and is often detrimental to the cultures. The addition of ethylene biosynthetic inhibitors such as silver nitrate, AVG (aminoethoxyvinylglycine), and silver thiosulphate have been shown to increase the formation of shoots.
- Cultures are grown in walk-in growth rooms or growth chambers. Humidity, light, and temperature must be controlled for the proper growth of cultures.
- A 16-hour light photoperiod is optimal for tissue cultures, and a temperature of 22 – 25ºC is used in most laboratories.
- Cool white fluorescent lamps also supply a light intensity of 25–50 µmol m-2 s-1.
- Relative humidity of 50–60% is maintained in the growth chambers. Some cultures are also incubated in the dark.
- Cultures can be cultivated in various containers, including test tubes, flasks, Petri dishes, and bottles.

Sterility

- The preservation of a sterile environment is necessary for effective tissue culture. The laminar flow hood is used for all tissue culture work. A dust filter and a high-efficiency particulate air (HEPA) filter are used in the laminar flow hood to filter the air. The hood must be kept spotless, which can be accomplished by wiping it with alcohol that contains 70% of the alcohol.
- The surfaces of plant tissues naturally contain a variety of bacteria and fungi. Before tissue culture, it is crucial to thoroughly clean the explant since contaminants can proliferate in the culture media.

They also compete with the plant tissue for nutrients, depriving it of those nutrients. Bacteria and fungi can rapidly surpass plant tissues and destroy them.

- Explants are commonly surface-sterilized using sodium hypochlorite, ethanol, and fungicides when using field-grown tissues.
- The type of tissue usually decides the time of sterilization. Leaf tissue requires a shorter sterilization time than seeds with a hard seed coat.

Plant Tissue Culture Types

Callus Culture

A callus is an unorganized mass of cells that develops when cells are wounded. When the explant is cultivated on media that promote the development of undifferentiated cells, a callus is formed. The majority of callus cells are formed with the aid of auxins and cytokinins. Using plant growth hormones, callus can multiply continuously or be directed to develop organs or somatic embryos.

Cell Suspension Culture

Small fragments of loose friable callus can be cultured as cell suspension cultures in a liquid medium. Cell suspensions can be maintained as batch cultures grown in flasks for long periods. A portion of callus tissue can be transferred into a liquid medium, and when subjected to continuous shaking, single-cell cultures and suspension cultures can be cultivated from callus cultures. The growth rate of the suspension-cultured cells is generally higher than that of the solid culture.

Anther/Microspore Culture

The culture of anthers or isolated microspores to produce haploid plants is known as anther or microspore culture. Embryos can be produced via a callus phase or be a direct recapitulation of the developmental stages characteristic of zygotic embryos. Compared to traditional breeding methods, microspore culture enables the creation of homozygous plants in a very short time. These homozygous plants are useful tools in plant breeding and genetic studies.

Protoplast Culture

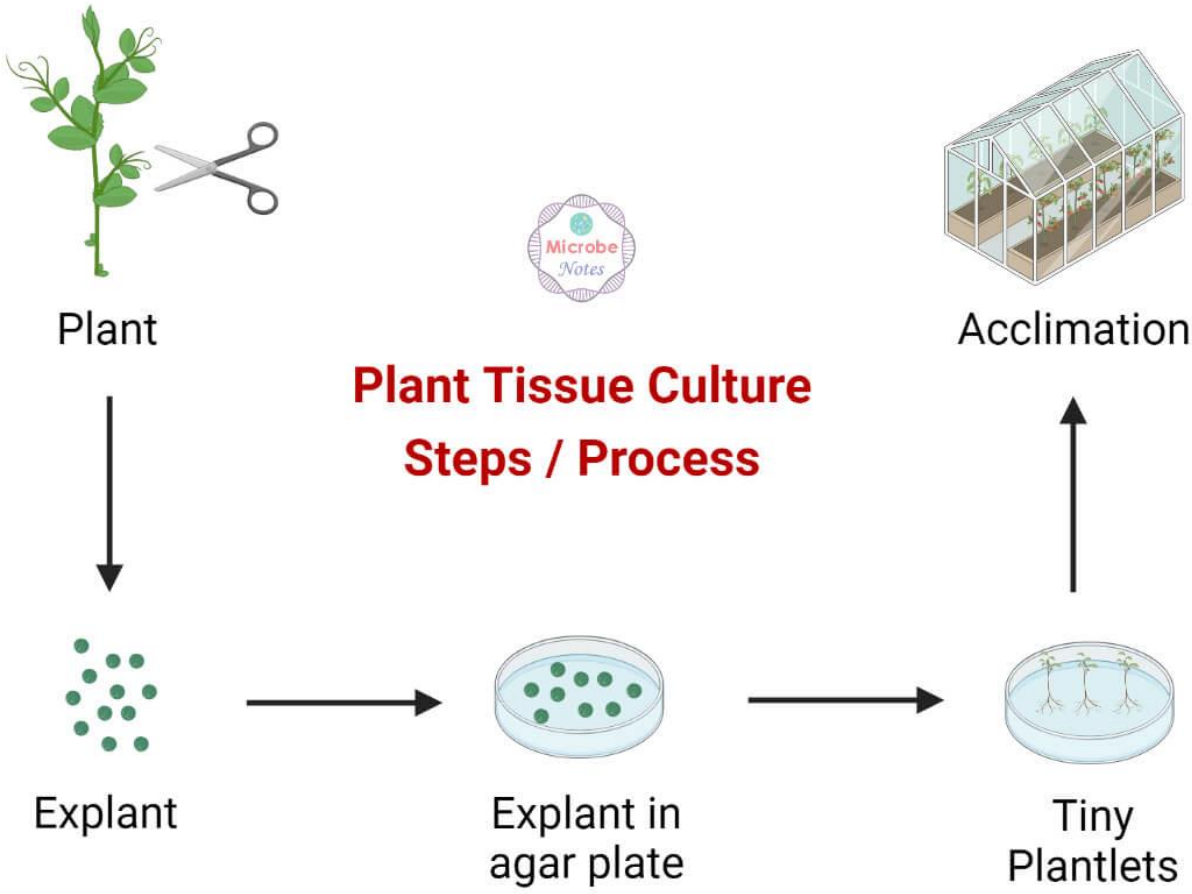
Protoplasts contain all the components of a plant cell except for the cell wall. Protoplasts can be used to create somatic hybrids and regenerate whole plants from a single cell. Cell walls of explant can be removed either mechanically or enzymatically. Protoplasts can be cultured either in liquid or solid medium. Protoplasts embedded in an alginate matrix and then cultured on a solid medium have better success rates of regeneration. Although protoplasts appear to be a very appealing method for regenerating plants and transferring genes, they are extremely delicate.

Embryo Culture

It is a technique in which isolated embryos from immature ovules or seeds are cultured in vitro. For species whose seeds are dormant, resistant, or prematurely sterile, embryo culture has been used as a helpful tool for direct regeneration. In plant breeding programs, embryo culture goes hand in hand with in vitro control of pollination and fertilization to ensure hybrid production. In addition, direct somatic embryos and embryogenic calluses can be produced from immature embryos.

Meristem Culture

Using apical meristem tips, it is possible to produce disease-free plants. This technique can be referred to as meristem culture, meristem tip culture, or shoot tip culture, depending on the actual explant used. Plant apical meristems make good explants for the cultivation of virus-free plants. Hence, this method is usually used to eliminate viruses in many species.



Plant Tissue Culture Procedure, Steps

Regeneration Methods of Plants in Culture

It includes two methods:

1. Organogenesis
2. Somatic Embryogenesis

Organogenesis

In plant tissue culture, it refers to the formation of either shoot or root. The equilibrium of auxin and cytokinin and the tissue's capacity to react to phytohormones during culture are key factors in in-vitro organogenesis. In-vitro organogenesis can be of two types:

1. Indirect organogenesis
2. Direct organogenesis

- Indirect organogenesis involves the formation of organs indirectly via a callus phase. To produce transgenic plants, induction of plants through a callus phase has been used. Either the callus is transformed, plants are regenerated, or the primary explant is transformed, and the callus is formed, and then shoots are cultivated from the explant. It is more important for transgenic plant production.
- Direct organogenesis involves direct bud or shoots formation from the tissue without a callus stage. Plants are usually propagated by direct organogenesis for improved multiplication rates and production of transgenic plants but mainly for clonal propagation.

Somatic Embryogenesis

Somatic embryogenesis is a nonsexual developmental process that produces a bipolar embryo with a closed vascular system from the somatic tissues of a plant. It has become one of the most powerful techniques in plant tissue culture for mass clonal propagation. Somatic embryogenesis may occur directly or via a callus phase. For clonal propagation, direct somatic embryogenesis is preferred since there is less chance of somaclonal mutation.

Indirect somatic embryogenesis is usually used in the selection of desired somaclonal variants and for the production of transgenic plants.

Encapsulated somatic embryos are known as synthetic seeds. Synthetic seeds have multiple advantages. They are easy to handle, they can potentially be stored for a long time, and there is potential for scaleup and low cost of production.

Rooting of shoots

The success of acclimatization of a plantlet greatly depends on root system production. Rooting of shoots can be achieved in vitro or ex-vitro.

Ex vitro rooting involves pretreating the shoots with phenols or auxins and then planting them directly in soil under high humidity, which significantly lowers the cost of manufacturing. This technique also allows simultaneous acclimation of the rooted shoots.

In vitro rooting consists of rooting the plants in axenic conditions. Despite the cost factor, in vitro rooting is still a common practice in many plant species.

Several factors are known to affect rooting. The most important factor is the action of endogenous and exogenous auxins. Phenolic compounds are also known to have a stimulatory effect on rooting. Phloroglucinol, a root promoter, is reported effective in root development. Catechol, a strong reducing agent, has been reported to regulate IAA oxidation.

Acclimation / Acclimatization

Once plants are generated by tissue culture, they have to be transferred to the greenhouse or field. This requires that the plants be hardened-off before transfer to the field. To reduce water loss during acclimatization, plants are initially transferred to a greenhouse or growth chamber. The relative humidity outside the vessels is often significantly lower than the humidity inside the vessels. Once the plants are acclimatized under greenhouse conditions, they are ready for transfer to the field.

Advantages of Plant Tissue Culture

1. Totipotency, nutrition, metabolism, division, differentiation, and preservation of plant cells.
2. Morphogenesis and plant regeneration from individual cells or tissues through organogenesis or somatic embryogenesis.
3. Variations were generated through in vitro culture.
4. Evolution of haploids through anther and pollen culture, including ovule culture.
5. Wide hybridization programs through ovule, ovary, and embryo cultures to overcome both pre-zygotic and post-zygotic sterility mechanisms.
6. Micropropagation of plant materials.
7. In vitro selection of mutants tolerant to biotic and abiotic stresses.
8. In vitro culture and secondary metabolite biosynthesis.
9. Plant genetic engineering using DNA transfer and in vitro culture techniques.

Disadvantages of Plant Tissue Culture

1. Labor-intensive and expensive process.
2. Vulnerable to many environmental factors.

UNIT III

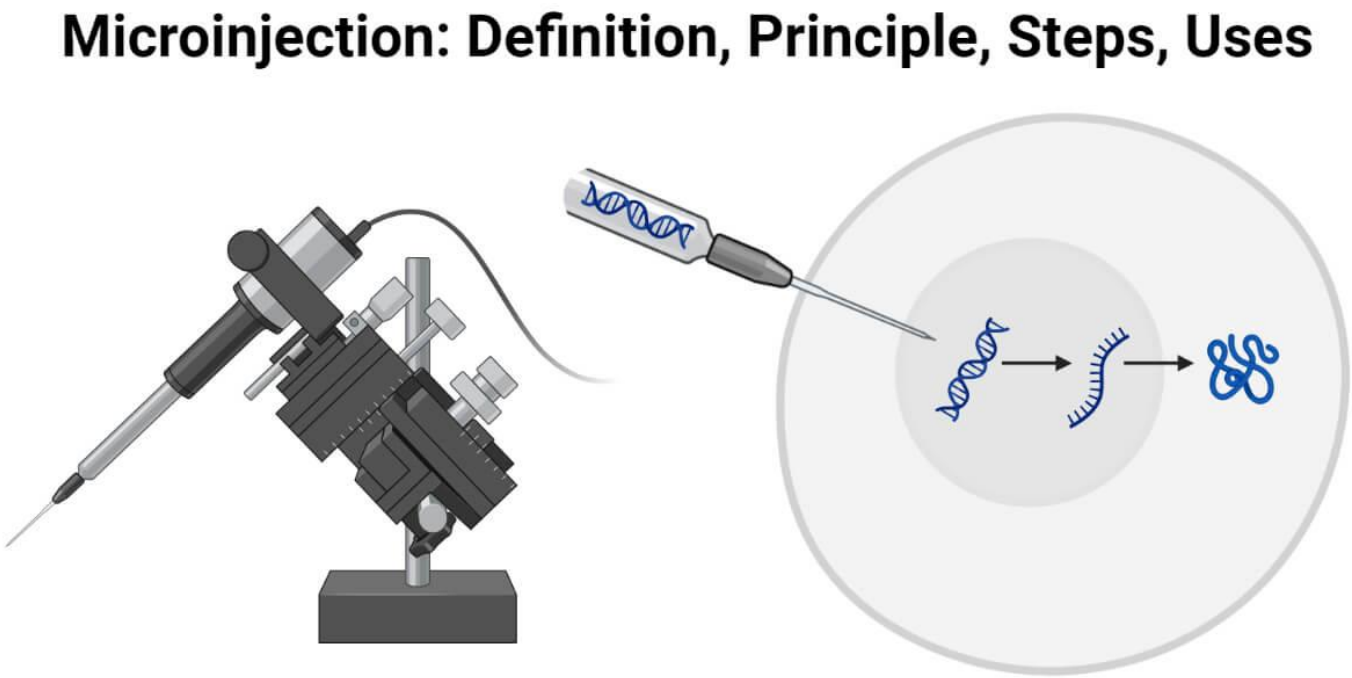
Microinjection: Definition, Principle, Steps, Uses

August 11, 2023 by Sanju Tamang

Edited By: Sagar Aryal

Microinjection is one of the physical methods of gene transfer used to introduce DNA or other genetic materials directly into a cell using a small glass needle or micropipette.

This method allows efficient transfer and integration of desired genes into the host cell's genome. It provides precise control over the delivery of desired materials making it a powerful tool in various research areas.

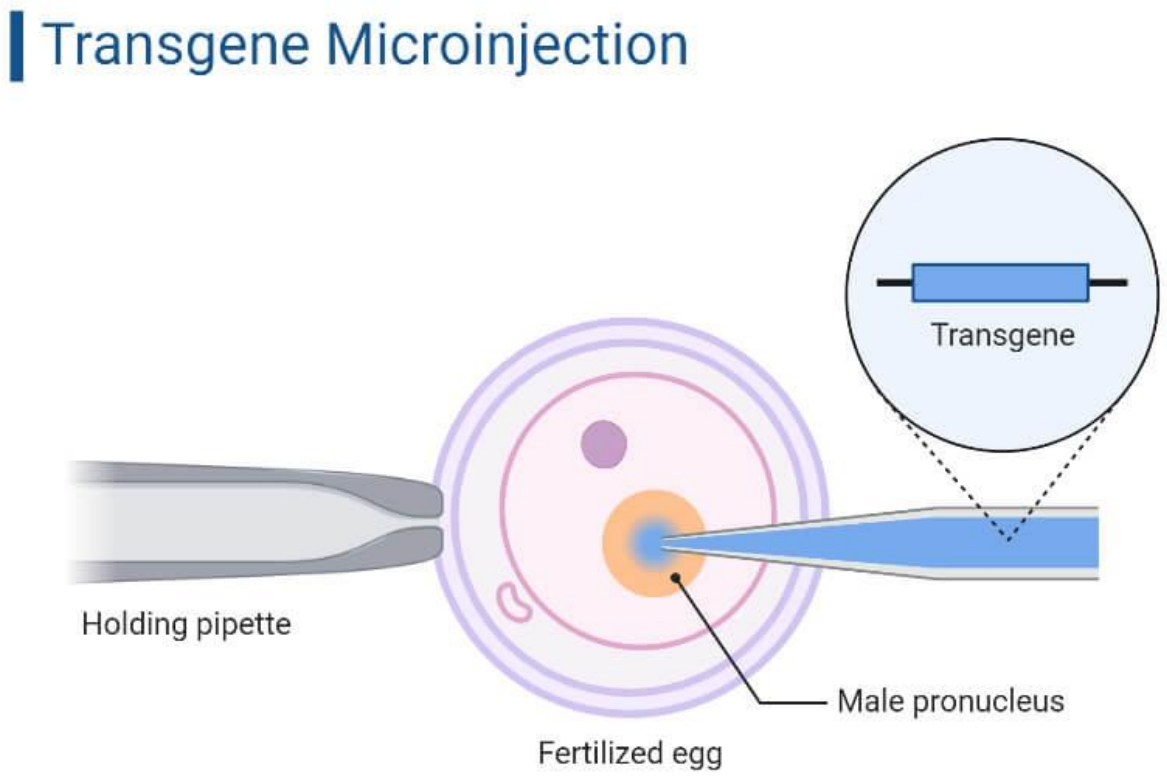


Microinjection

Dr. Marshall A. Barber introduced the concept of DNA microinjection during the early 19th century. Since then, it has continually evolved to keep up with the advancements in biomedical fields. It has applications in diverse areas such as transgenics, animal cloning, human infertility treatment, genetic engineering, and genome editing.

Principle of Microinjection

The principle of microinjection is based on the direct delivery of genetic material into individual cells using a fine glass needle called a micropipette, a positioning device known as a micromanipulator, and a microinjector. The process is performed under a powerful microscope. The genetic material is delivered into the cell by applying hydrostatic pressure to release a fluid containing the DNA through the micropipette. The small tip diameter of the micropipette and the precise movements enabled by the micromanipulator allows the precise delivery of desired materials.

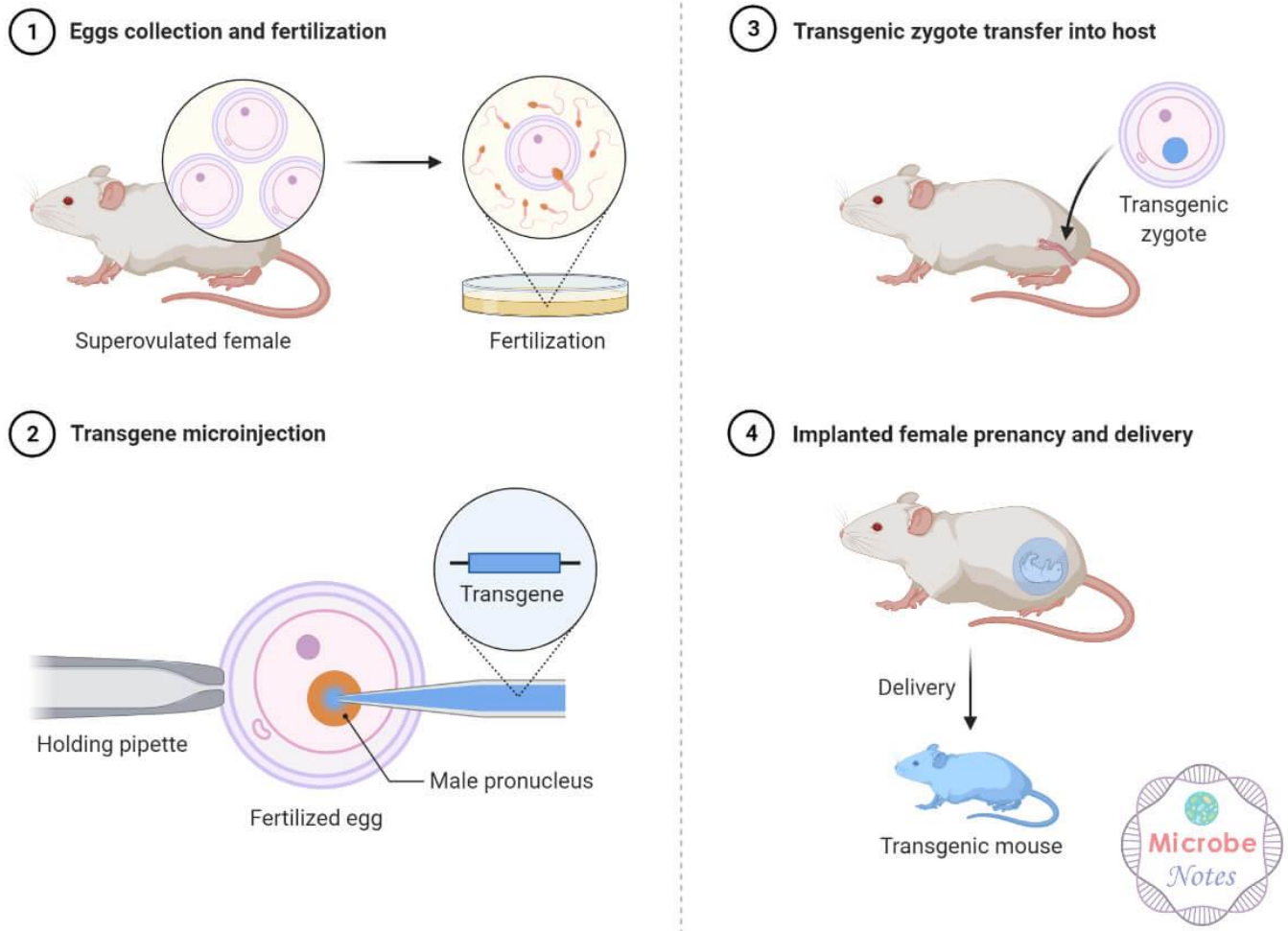


Transgene Microinjection

Steps in Microinjection

1. First, the glass micropipette or needle is prepared by heating and stretching the glass to form a fine tip at the heated end. The resulting tip size is usually around 0.5 mm in diameter, resembling an injection needle.
2. The entire process of delivering foreign materials using microinjection is performed under a powerful microscope for precise manipulation and observation.
3. The cells to be microinjected are placed in a container.
4. A holding pipette is placed near the target cell. It uses gentle suction to hold the cell during the injection process.
5. The micropipette, containing the desired contents, is mounted on a micromanipulator, which allows precise positioning and movement of the pipette. The micropipette is lowered near the cell.
6. The micropipette is carefully inserted into the cell membrane, either into the cytoplasm or the nucleus, depending on the target location for the genetic material. The contents of the micropipette are then injected into the cell by applying hydrostatic pressure using a microinjector. The pressure forces the fluid containing the genetic material to be released through the micropipette into the cell.

7. After delivering the contents into the cell, the empty micropipette needle is slowly and carefully withdrawn from the cell, minimizing damage to the cell and its membrane. Proper technique and high-quality micropipettes help minimize cell death during this step.



Transgenic Mice Production-Microinjection

Applications of Microinjection

Some major applications of microinjection are:

- Microinjection is commonly used in the production of transgenic animals, introducing foreign DNA into fertilized eggs to study gene function and create disease models.
- Microinjection was used to produce the first chimeric transgenic mice. Chimeric animals have been useful in studying embryonic development, tissue transplantation, and understanding cell lineage.
- In the field of in vitro fertilization, microinjection is used in intracytoplasmic sperm injection (ICSI), allowing successful fertilization in cases of male infertility, and leading to term pregnancies and healthy births. In ICSI, individual sperm with motility defects are microinjected directly into isolated oocytes.
- Microinjection is also used in somatic cell nuclear transfer (SCNT) to create genetically identical copies of an organism by transferring a somatic cell into an enucleated oocyte.

- Microinjection has been widely used in various studies, including human embryo research and injection of mitotic cells.
- In neuroscience, microinjection is useful for working with primary cultured human neurons, delivering proteins, peptides, and cDNA constructs into the cytosol of human neurons which is difficult to perform using other gene transfer techniques.

Advantages of Microinjection

- Unlike other gene transfer methods, microinjection does not require the use of selection markers such as antibiotic-resistance genes. This makes the process easier and removes the need for additional steps to identify and isolate transformed cells.
- Microinjection allows precise delivery of materials in terms of volume and timing, which can be challenging with other methods like electroporation or transfection.
- Injected cells can be easily identified by co-injecting a marker dye or fluorescently labelled proteins.
- Microinjection requires less protein preparation compared to electroporation, making it advantageous for experiments involving less abundant or expensive proteins and peptides.
- Microinjection is less stressful to cells, reducing cell death that is commonly observed with chemical transfection or viral infection methods.

Disadvantages of Microinjection

- Microinjection requires vehicle controls to assess potential effects on cell viability and to ensure accurate results.
- The technical expertise required to master the microinjection method and maintain cell viability is another limitation of this method.
- The use of alternative techniques like transfection, infection, and electroporation has become more popular compared to microinjection for delivering non-permeable foreign materials into single cells.
- Manual microinjection is labour-intensive and time-consuming, making it less practical for large-scale experiments or high-throughput applications.
- Microinjection typically involves injecting a small population of cells within a larger cell culture, which can limit scalability.
- Microinjection is not an appropriate method for transferring genetic material into a large number of cells for techniques like Western blotting or purification.
- Direct microinjection of certain proteins, like membrane proteins or neurotransmitter receptors, is challenging.

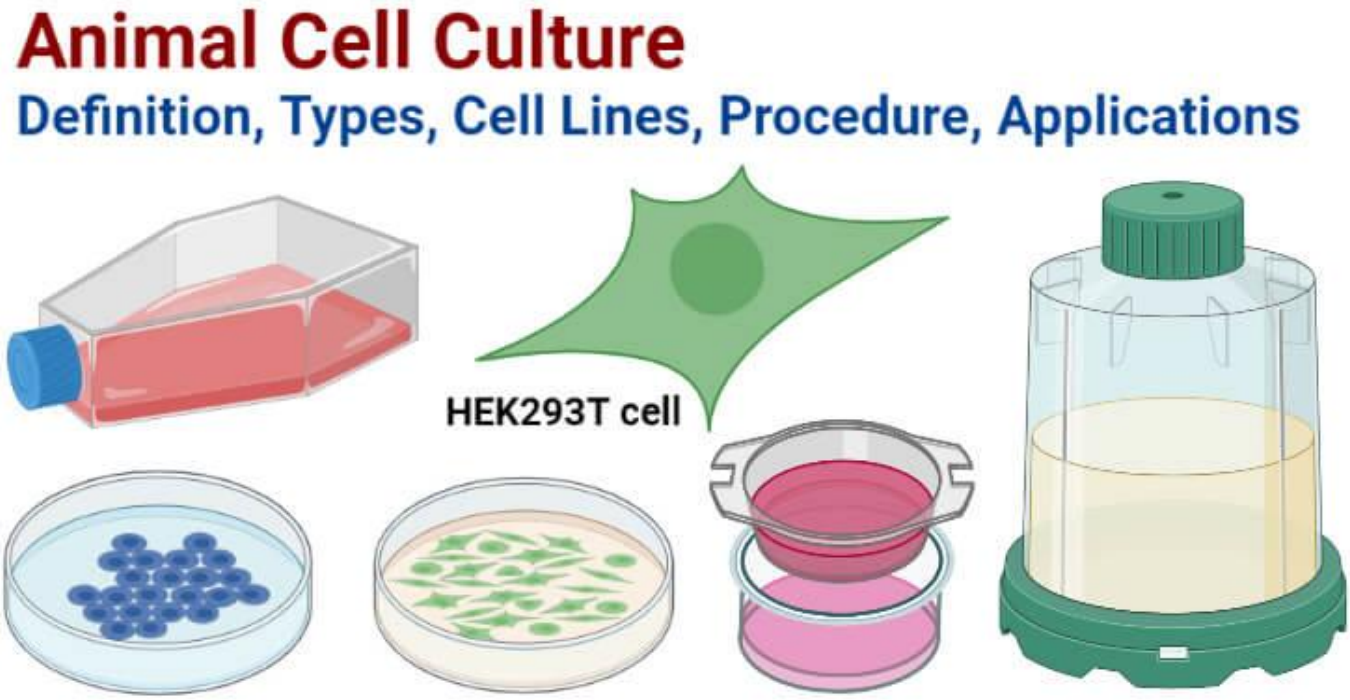
Animal Cell Culture: Types, Cell Lines, Procedure, Uses

May 2, 2024 by Anupama Sapkota

Edited By: Sagar Aryal

Animal cell culture is a type of biotechnological technique where animal cells are artificially grown in a favorable environment.

- The cells used in animal cell culture are usually obtained from multicellular eukaryotes and their established cell lines.
- Animal cell culture is a common and widely used technique for the isolation of cells and their culture under artificial conditions.
- This technique was developed as a laboratory technique for particular studies; however, it has since been developed to maintain live cell lines as a separate entity from the original source.
- The development of animal cell culture techniques is due to the development of basic tissue culture media, which enables the working of a wide variety of cells under different conditions.
- In vitro culture of isolated cells from different animals has helped in the discovery of different functions and mechanisms of operations of different cells.
- Some of the areas where animal cell culture has found most applications include cancer research, vaccine production, and gene therapy.
- The growth of animal cells on artificial media is difficult than growing microorganisms on artificial media and thus, require more nutrients and growth factors.
- However, advances in the culture media have made it possible to culture both undifferentiated and differentiated cells on artificial media.
- Animal cell cultures can be performed from different complexities of cells as complex structures like organs can also be used to initiate organ culture in vitro.
- Depending on the purpose and application of the technique, cells, tissues, or organs can be used for the culture process.



Animal Cell Culture. Created with BioRender.com

Types of Animal cell culture

Animal cell cultures can be divided into two distinct groups depending on the number of cell divisions occurring during the process;

1. Primary cell culture

- Primary cell culture is the first culture obtained directly from animal tissue via mechanical and chemical disintegration or enzymatic methods.
- The cells of the primary cell culture are slow-growing cells that carry all the characteristics of the original tissue or cells.
- Since these cultures are obtained directly from the origin, they have the same number of chromosomes as the original cells.
- Primary cell cultures are performed in order to preserve and maintain the growth of cells on an artificial growth medium at a particular condition.
- Primary cell cultures can be subcultured to obtained other cultures that either continue to grow indefinitely or die after a few subcultures.
- The subsequent subculture of primary cell culture results in the introduction of mutations into the cells, which might result in cell lines.
- The morphology of cells in the primary cell cultures might be different and varied, with the most observed morphological structures being epithelium type, epithelioid type, fibroblast type, and connective tissue type.

- Primary cell cultures are difficult to obtain and usually have a shorter lifespan. Besides, these are prone to contamination by bacteria and viruses.
- The increase in cell numbers in the primary cell culture can result in exhaustion of the substrate and nutrients, which affects cellular activity.
- Usually, primary cell cultures need to be subcultured in order to maintain continuous cell growth once they reach the confluence stage.

Primary cell cultures can be further divided into two groups depending on the kind of cells present in the culture;

a. Anchorage-dependent/Adherent cells

- The cells in the culture require a stable biologically inert surface for adherence and growth.
- The surface should be solid and nontoxic as these cells are difficult to grow as cell suspensions.
- These cells are usually obtained from the tissues of organs where the cells remain immobilized within the connective tissue.
- Examples of adherent cells include kidney cells and mouse fibroblast STO cells.

b. Anchorage-independent/ Suspension cells

- These cells can grow efficiently as cell suspensions and do not require a solid surface for attachment.
- These can be grown on liquid media continuously to obtain fresh subcultures.
- The ability of the cells to grow as suspension depends on the source of cells as cells that remain as suspensions in the body are effective suspension cells.
- Examples of suspension cells include blood cells that are vascular and remain suspended in the plasma.

2. Secondary cell culture

- Secondary cell cultures are obtained after the primary cell cultures are subsequently subcultured over a period of time in fresh culture media.
- The cells of the secondary cell cultures are long-lasting as these have a higher lifespan due to the availability of appropriate nutrients at regular intervals of time.
- Secondary cell cultures are favored over primary cell cultures as these are more readily available and are easy to grow and preserve.
- These are formed from the enzymatic treatment of the adherent cells followed by washing and resuspension of cells in particular volumes of fresh media.
- Secondary cell cultures are prepared when the number of cells in the primary culture exceeds the capacity of the medium to support growth.
- Secondary cultures help to maintain an optimal cell density necessary for continued growth.

- The cells of the secondary cell culture might not resemble that on the parental tissue as mutations, and genetic alterations might be introduced during the subculture process.
- The cells can be transformed as, in some cases, the continuous subculture can lead to immortal cells.
- The risk of contamination by bacteria and viruses is less as the cells transform and become less susceptible to infections.
- An important disadvantage associated with secondary cell culture is that the cells might develop the tendency to differentiate over a long period of time and result in aberrant cells.

Cell Lines

A cell line is a group of cells that are formed from the subculture of primary culture consisting of a pure culture of cells. Cell lines usually display functional features that are close to the primary cells, but the genotype and phenotype of the cells can be modified. A cell line consists of several cell lineages with similar or different phenotypes.

Cell lines can be further divided into two groups based on the growth patterns of the cells;

a. Finite cell lines

- Finite cell lines are cell lines where the cells in the culture divide for a limited number of times, after which they eventually die.
- The cells in the finite cell lines can divide from 20 to 100 times before they eventually die and cannot divide anymore.
- The number of cell division and lifespan depends on a number of factors like cell lineage differences, species, culture conditions, and media.
- The cells of the finite cell lines grow as adherent cells on solid surfaces.

b. Continuous cell lines

- Continuous cell lines are cells that exhibit indefinite growth via subsequent subcultures.
- The cells in the continuous cell lines grow faster to form an independent culture. The cells are immortal and can divide indefinitely.
- The cells in the continuous cell lines can be transformed via genetic alterations and are also tumorigenic.
- The transformed cells are formed from the normal primary cell cultures after treatment with chemical carcinogens or by infection with oncogenic viruses.
- The cells are capable of growing to prepare higher cell density and can grow as suspensions on liquid media.
- These cells can even grow on top of each other to form multilayered structures on the culture vessels.

Examples of common Cell Lines

The following are some of the common examples of cell lines;

a. HeLa cell line

- HeLa cells are one of the first continuous culture human cell lines with the help of cells of the cervical carcinoma.
- These cells are used for processes like virus cultivation and preclinical drug evaluation.

b. HL 60 (Leukemia)

c. MCF-7 (breast cancer cells)

Procedure or Protocol of Animal cell culture

1. Growth Conditions

- Animal cell culture requires the use of specific culture media that are more complex and specific than the basic culture media used for microbial growth.
- Some of the important basic components of the media are inorganic salts, nitrogen source, energy source, vitamins, fat and fat-soluble vitamins, growth factors, and hormones. In some cases, pH buffering systems and antibiotics are also added.
- The temperature for the growth depends on the source of the cell as different organisms require different temperatures for cell growth and division.
- Warm-blooded animal cells can be cultured at 37°C as the optimal temperature, whereas cold-blooded animals grow between 15°C-25°C.

2. Primary cell culture

- Primary cell cultures are obtained from fresh tissues that are removed from the organs with the help of an aseptic razor.
- In some cases, the cells are removed by the use of chemical disintegrators or proteolytic enzymes.
- The cell suspension obtained is washed with buffering liquid in order to remove the proteolytic enzymes.
- The cell suspension is poured onto a flat surface which can be a culture vessel or a sterile Petri plate.
- The cells that can adhere to the base of the vessel are overlaid with an appropriate culture medium and incubated at room temperature.

3. Cell thawing

- In the case of subsequent subcultures, the preserved cell culture might have to be used.
- The water bath is heated to a temperature of 37°C, and the growth media where the cells are to be plated is warmed.
- The warm medium is added to the culture vessel. The vial with the frozen cells is then placed in the water bath until thawed.

- After thawing, the via is washed with 70% alcohol on the outside. The cell suspension is pipetted into the cell culture vessel and swirled gently to mix everything.
- The medium is then incubated overnight under the usual growth conditions. The growth medium is replaced the next day.

4. Trypsinizing Cells

- Trypsinization is the method of separating adherent cells from the surface of the culture vessel with the help of proteolytic enzymes. It is done when the cells are to be used for passaging, counting, or other purposes.
- The medium is removed, and the cells are recovered. The cells are then washed with phosphate buffer.
- Warm trypsin-EDTA is added to the vessel so as to cover the monolayer. The vessel can be rocked to ensure that the monolayer is coated.
- The vessel is incubated in a CO_2 incubator at 37°C for 1-3 minutes.
- The vessel is removed from the incubator, and the flask is firmly tapped on the side with the palm of the hand to assist detachment.
- Once the cells are dislodged, they are resuspended in an appropriate growth medium containing some amount of serum.
- The cells are then separated with the help of syringe needles by disrupting the cell clumps and used accordingly.

Applications of Animal cell culture

The following are some of the applications of animal cell culture;

a. Production of vaccines

- Animal cell culture is an important technique used for the development of viral vaccine production.
- The technique has been used for the development of a recombinant vaccine against hepatitis B and poliovirus.
- Immortalized cell lines are used for the large-scale or industrial production of viral vaccines.

b. Recombinant proteins

- Animal cell cultures can also be used to produce recombinant therapeutic proteins like cytokines, hematopoietic growth factors, growth factors, hormones, blood products, and enzymes.
- Some of the common animal cell lines used to produce these proteins are baby hamster kidney and CHO cells.

c. Gene Therapy

- The development of animal cell culture is critical for the advances in gene therapy.

- Cells with faulty genes can be replaced by a functional gene in order to remove such defects and diseases.

d. Model systems

- Cells obtained from cell culture can be studied as a model system for studies related to cell biology, host-pathogen interactions, effects of drugs, and effects due to changes in the cell composition.

e. Cancer Research

- Animal cell culture can be used to study the differences in cancer cells and normal cells as cancer cells can also be cultured.
- The differences allow more detailed studies on the potential causes and effects of different carcinogenic substances.
- Normal cells can be culture to form cancer cells by the use of certain chemicals, viruses, and radiation.
- Cancer cells can also be used as test systems for studies related to the efficiencies of drugs and techniques used in cancer treatment.

f. Production of Biopesticides

- Animal cell lines like Sf21 and Sf9 can be used to produce biopesticides due to their faster growth rate and higher cell density.
- Organisms like baculovirus can be produced through animal cell culture as well.

Advantages of Animal cell culture

The following are some of the advantages of animal cell culture;

1. Cell culture is superior to other similar biotechnological approaches as it allows the alteration of different physiological and physiobiological conditions like temperature, pH, and osmotic pressure.
2. Animal cell culture enables studies related to cell metabolism and understand the biochemistry of cells.
3. It also allows observation of the effects of various compounds like proteins and drugs on different cell types.
4. The results from animal cell cultures are consistent if a single cell type is used.
5. The technique also enables the identification of different cell types on the basis of the presence of markers like molecules or by karyotyping.
6. The use of animal cell culture for testing and other processes prevents the use of animals in experiments.
7. Animal cell culture can be used to produce large quantities of proteins and antibodies, which would otherwise require a large investment.

Disadvantages of Animal cell culture

Even though animal cell culture has been used as a technologically advanced method, there are some disadvantages associated with this approach.

1. It is a specialized technique that requires trained personnel and aseptic conditions. The technique is an expensive process as it requires costly equipment.
2. The subsequent subculture of the cell culture might results in differentiated properties as compared to the original strain.
3. The method produces a minuscule amount of recombinant proteins, which further increases the expenses of the process.
4. Contamination with mycoplasma and viral infection occur frequently and are difficult to detect and treat.
5. The cells produced by this technique lead to instability due to the occurrence of aneuploidy chromosomal constitution.

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it to be incorporated into the DNA of the host and to be expressed correctly by the cells of the host. Transgenic sheep and goats have been produced those express foreign proteins in their milk. Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs. These animals should eventually prove to be valuable sources of proteins for human therapy.

Note

In July 2000, researchers from the team that produced Dolly reported success in producing transgenic lambs in which the transgene had been inserted at a specific site in the genome and functioned well.

Transgenic **mice** have provided the tools for exploring many biological questions.

Example

Normal mice cannot be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily-manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

- can be infected by polio virus and even
- develop paralysis and other pathological changes characteristic of the disease in humans.

Two methods of producing transgenic mice are widely used:

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

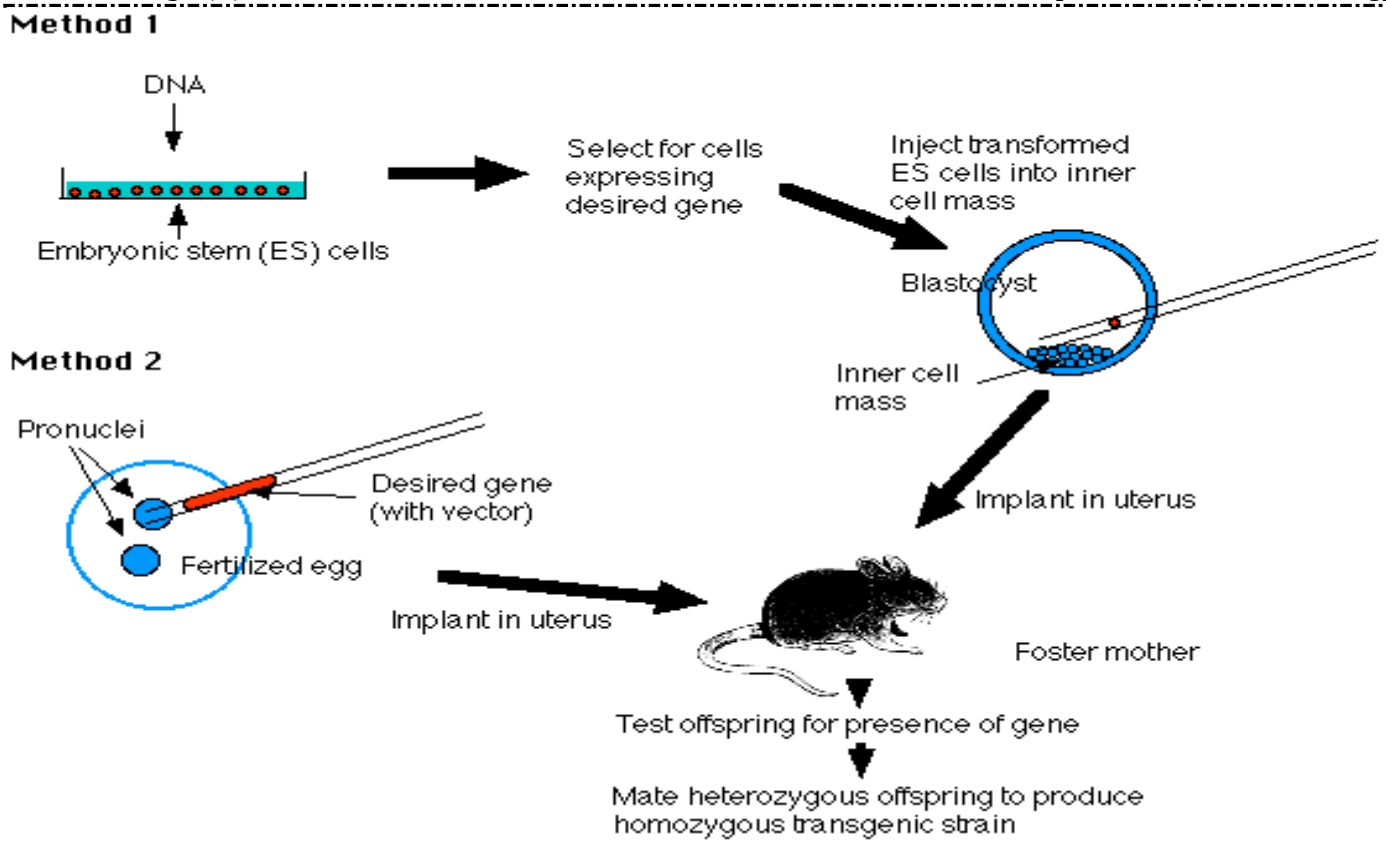


Fig.11.4.1 Methods to produce Transgenic mice

The Embryonic Stem Cell Method - Method 1

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

1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

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

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells

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

5. Embryo transfer

- Prepare a **pseudopregnant** mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.

- Transfer the embryos into her uterus.
- Hope that they **implant** successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

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

7. Establish a transgenic strain

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

The Pronucleus Method - Method 2

1. Prepare your DNA as in Method 1

2. Transform fertilized eggs

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

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

Example

This image (courtesy of R. L. Brinster and R. E. Hammer) shows a transgenic mouse (right) with a normal littermate (left). The giant mouse developed from a fertilized egg transformed with a recombinant DNA molecule containing:

- the gene for **human growth hormone**
- a strong mouse gene **promoter**

The levels of growth hormone in the serum of some of the transgenic mice were several hundred times higher than in control mice.

Random vs. Targeted Gene Insertion

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

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

In either case, targeted gene insertion requires

- the desired gene
- ***neo***^{r}, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells
- ***tk***, a gene that encodes **thymidine kinase**, an enzyme that phosphorylates the nucleoside analog **ganciclovir**. **DNA polymerase** fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So ganciclovir kills cells that contain the *tk* gene

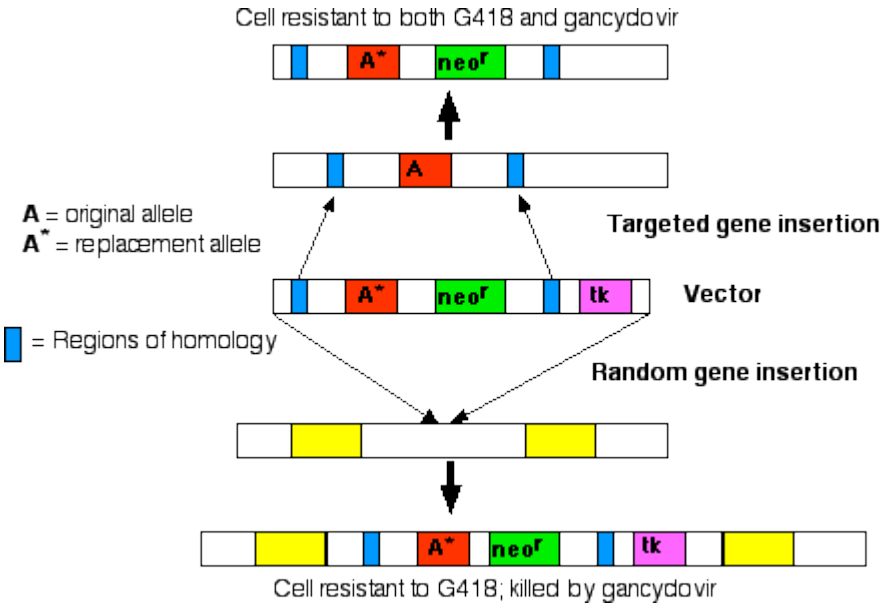


Figure 11.4.2 Neo vector

Step 1

Treat culture of ES cells with preparation of vector DNA.

Results:

- **Most cells** fail to take up the vector; these cells will be killed if exposed to G418.
- In a **few cells**: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the ***tk*** gene, is inserted into host DNA. These cells are resistant to G418 but killed by gancyclovir.
- In **still fewer cells**: **homologous recombination** occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome, and the region between these homologous sequences replaces the equivalent region in the host DNA.

Step 2

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

- The cells (the majority) that failed to take up the vector are killed by G418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the *tk* gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).

Step 3

Inject these into the inner cell mass of mouse blastocysts.

Knockout Mice: What do they teach us?

If the replacement gene (**A*** in the diagram) is nonfunctional (a "null" allele), mating of the heterozygous transgenic mice will produce a strain of "**knockout mice**" homozygous for the nonfunctional gene (both copies of the gene at that locus have been "knocked out"). Knockout mice are valuable tools for discovering the function(s) of genes for which mutant strains were not previously available. Two generalizations have emerged from examining knockout mice:

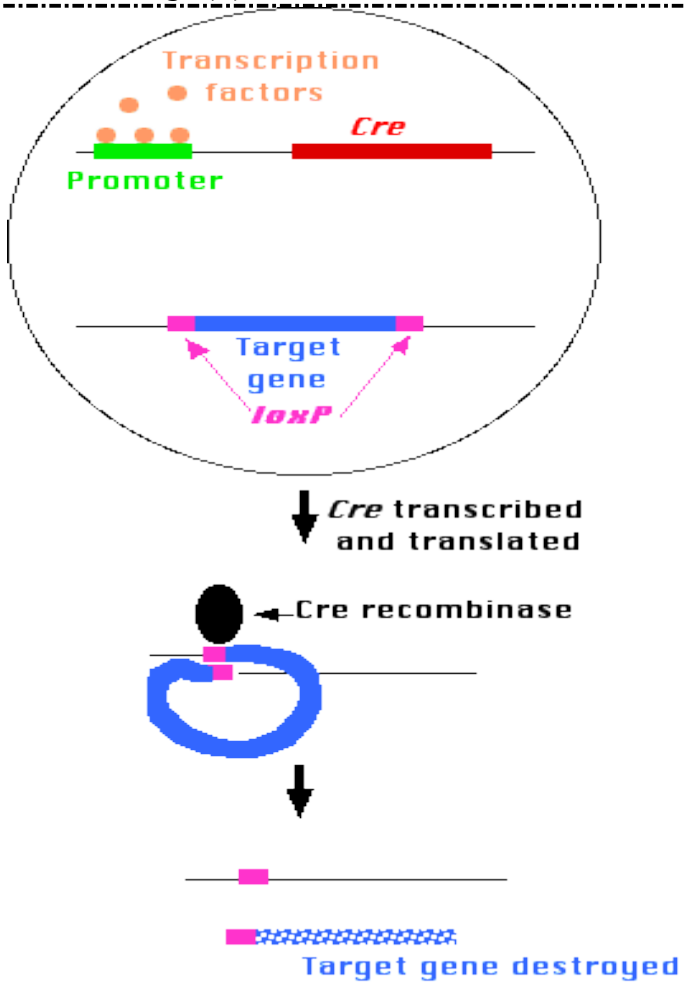
- Knockout mice are often surprisingly unaffected by their deficiency. Many genes turn out not to be indispensable. The mouse genome appears to have sufficient redundancy to compensate for a single missing pair of alleles.
- Most genes are **pleiotropic**. They are expressed in different tissues in different ways and at different times in development.

Tissue-Specific Knockout Mice

While "housekeeping" genes are expressed in all types of cells at all stages of development, other genes are normally expressed in only certain types of cells when turned on by the appropriate signals (e.g. the arrival of a hormone).

To study such genes, one might expect that the methods described above would work. However, it turns out that genes that are only expressed in certain adult tissues may nonetheless be vital during embryonic development. In such cases, the animals do not survive long enough for their knockout gene to be studied. Fortunately, there are now techniques with which transgenic mice can be made where a particular gene gets knocked out in only one type of cell.

The Cre/*loxP* System



The Cre/loxP System

One of the bacteriophages that infects E. coli, called P1, produces an enzyme — designated Cre — that cuts its DNA into lengths suitable for packaging into fresh virus particles. Cre cuts the viral DNA wherever it encounters a pair of sequences designated *loxP*. All the DNA between the two *loxP* sites is removed, and the remaining DNA ligated together again (so the enzyme is a recombinase). Using "Method 1" above, mice can be made transgenic for

- the gene encoding Cre attached to a promoter that will be activated only when it is bound by the same transcription factors that turn on the other genes required for the unique function(s) of that type of cell;
- a "target" gene, the one whose function is to be studied, flanked by *loxP* sequences.

In the adult animal,

- those cells that
    - receive signals (e.g., the arrival of a hormone or cytokine)
    - to turn on production of the transcription factors needed
    - to activate the promoters of the genes whose products are needed by that particular kind of cell

will also turn on transcription of the Cre gene. Its protein will then remove the "target" gene under study.

- All other cells will lack the transcription factors needed to bind to the Cre promoter (and/or any enhancers) so the target gene remains intact.

The result: a mouse with a particular gene knocked out in only certain cells.

Knock-in Mice

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

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

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

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.
3. 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).

4. Promoter sites from the **beta-lactoglobulin** gene. These promote hormone-driven gene expression in milk-producing cells.
5. Binding sites for ribosomes for efficient translation of the beta-lactoglobulin 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 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 Chickens

Chickens grow faster than sheep and goats and large numbers can be grown in close quarters. They also 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.

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.

Transgenic Primates

In the 28 May 2009 issue of **Nature**, Japanese scientists reported success in creating transgenic marmosets. Marmosets are primates and thus our closest relatives (so far) to be genetically engineered. In some cases, the transgene (for green fluorescent protein) was incorporated into the germline and passed on to the animal's offspring. The hope is that these transgenic animals will provide the best model yet for studying human disease and possible therapies.

UNIT V

Bioethics

Bioethics in Cloning and Stem Cell Research

Cloning:

- **Reproductive Cloning**: Creating a genetic duplicate of an organism. Ethical concerns include the potential for reduced genetic diversity, identity issues, and the welfare of clones.
- **Therapeutic Cloning**: Generating embryonic stem cells for research and treatment of diseases. Ethical issues include the destruction of embryos and potential for exploitation.

Stem Cell Research:

- **Embryonic Stem Cells**: Derived from embryos, these cells can differentiate into any cell type. Ethical issues include the moral status of embryos and concerns about consent.
- **Adult Stem Cells**: Found in adult tissues, these cells have limited differentiation potential. Ethical debates focus on the balance between scientific advancement and respecting human life.

Human and Animal Experimentation

Human Experimentation:

- **Informed Consent**: Ensuring participants are fully informed about the study and its risks.
- **Risk-Benefit Analysis**: Weighing the potential benefits of research against the risks to participants.
- **Privacy and Confidentiality**: Protecting the personal information of participants.

Animal Experimentation:

- **3Rs Principle**: Replacement (using alternatives to animals), Reduction (using fewer animals), and Refinement (minimizing pain and distress).
- **Animal Welfare**: Ensuring humane treatment of animals in research, including proper housing, nutrition, and medical care.
- **Regulations**: Compliance with laws and guidelines governing animal research, such as the Animal Welfare Act.

Animal Rights/Welfare

- **Animal Rights**: The belief that animals have inherent value and should be treated with respect, advocating for the abolition of animal use in research, entertainment, and food.
- **Animal Welfare**: The focus on ensuring that animals used by humans are treated humanely and ethically, with minimal suffering and good living conditions.

Biosafety

Introduction to Biological Safety Cabinets

- **Class I Cabinets**: Provide personnel and environmental protection but no product protection.
- **Class II Cabinets**: Offer personnel, environmental, and product protection, commonly used in microbiological work.
- **Class III Cabinets**: Provide maximum protection for personnel, environment, and product, used for high-risk pathogens.

Primary Containment for Biohazards

- **Containment Equipment**: Biological safety cabinets, sealed centrifuge rotors, and safety containers.
- **Personal Protective Equipment (PPE)**: Gloves, lab coats, face shields, and respirators.

Biosafety Levels

- **BSL-1**: Work with low-risk microbes; minimal containment.
- **BSL-2**: Work with moderate-risk pathogens; includes restricted access and use of PPE.
- **BSL-3**: Work with high-risk airborne pathogens; requires controlled access, specialized ventilation.
- **BSL-4**: Work with high-risk, life-threatening pathogens; involves full-body, air-supplied suits, and highly controlled lab access.

Good Laboratory Practice (GLP)

- **GLP Principles**: Ensures the quality, reliability, and integrity of non-clinical safety data.
- **Components**: Proper documentation, trained personnel, validated methods, and well-maintained equipment.

Good Manufacturing Practice (GMP)

- **GMP Standards**: Ensures products are consistently produced and controlled according to quality standards.
- **Key Elements**: Quality management, proper documentation, quality raw materials, validated processes, and effective quality control.

Intellectual Property Rights (IPR)

Introduction to Intellectual Property (IP)

- **Intellectual Property**: Legal rights granted to creators for their inventions, literary and artistic works, and symbols, names, and images used in commerce.

Types of IP

Patents:

- **Definition**: Exclusive rights granted for an invention, allowing the patent holder to exclude others from making, using, or selling the invention for a limited period.
- **Criteria**: Novelty, non-obviousness, and industrial applicability.

- **Duration**: Generally, 20 years from the filing date.

Trademarks:

- **Definition**: A symbol, word, or phrase legally registered or established by use as representing a company or product.
- **Purpose**: Distinguishes goods or services of one enterprise from those of others.
- **Duration**: Can last indefinitely with proper use and renewal.

Copyright:

- **Definition**: Legal right that grants the creator of original works exclusive rights to their use and distribution.
- **Protected Works**: Literary, musical, and artistic works, including software.
- **Duration**: Typically, the lifetime of the author plus 70 years.