MODULE 8- LECTURE 1

GENE THERAPY: INTRODUCTION AND METHODS

8-1.1 Introduction

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery. In 1990 FDA for the first time approved a gene therapy experiment on ADA-SCID in the United States after the treatment of Ashanti DeSilva. After that, approximately 1700 clinical trials on patients have been performed with various techniques and genes for numerous diseases. Many diseases such as ADA-SCID, X-linked SCID, Leber's congenital amaurosis (a retinal disease), Parkinson's disease, multiple myeloma, chronic and acute lymphocytic leukemia, adrenoleukodystrophy have reported of successful clinical trials. But these are still not approved by FDA. Some other diseases on which gene therapy based research is going on are Haemophilia, Tyrosinemia, Hyperbilirubinemia (Criglar-Nijjar Syndrom), Cystic Fibrosis and many other cancers. After 30 years of research and clinical trials, only one product called Glybera got approval in November 2012 which may be available in market in late 2013. It has the ability to cure lipoprotein lipase deficiency (LPLD) a very rare disease.

8-1.2 Types of gene therapy

There are several approaches for correcting faulty genes; the most common being the insertion of a normal gene into a specific location within the genome to replace a non functional gene. Gene therapy is classified into the following two types:

1. Somatic gene therapy

2. Germ line gene therapy

8-1.2 .1 Somatic Gene Therapy

In somatic gene therapy, the somatic cells of a patient are targeted for foreign gene transfer. In this case the effects caused by the foreign gene is restricted to the individual patient only, and not inherited by the patient's offspring or later generations.

8-1.2 .1 Germ Line Gene Therapy

Here, the functional genes, which are to be integrated into the genomes, are inserted in the germ cells, i.e., sperm or eggs. Targeting of germ cells makes the therapy heritable.

8-1.3 Gene Therapy Strategies

8-1.3.1 Gene Augmentation Therapy (GAT)

In GAT, simple addition of functional alleles is used to treat inherited disorders caused by genetic deficiency of a gene product, e.g. GAT has been applied to autosomal recessive disorders. Dominantly inherited disorders are much less amenable to GAT. Figure 8-1.3.1 shows the GAT strategy

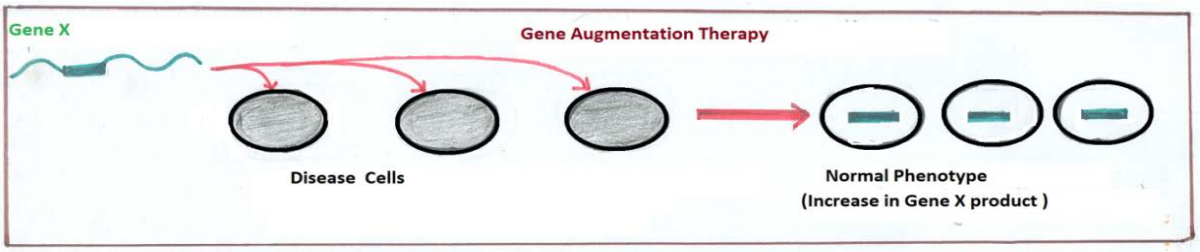

Figure 8-1.3.1: A gene therapy vector has been designed to treat the diseased cells with a gene X. This vector was introduced inside the diseased cells by various gene transfer methods. After a successful homologous recombination the treated cells will show the presence of gene X product as well as normal phenotype.

8-1.3.2 Targeted Killing of Specific Cells

It involves utilizing genes encoding toxic compounds (**suicide genes**), or **prodrugs** (reagents which confer sensitivity to subsequent treatment with a drug) to kill the transfected/ transformed cells. This general approach is popular in cancer gene therapies. This is shown in figure 8-1.3.2a & 8-1.3.2b

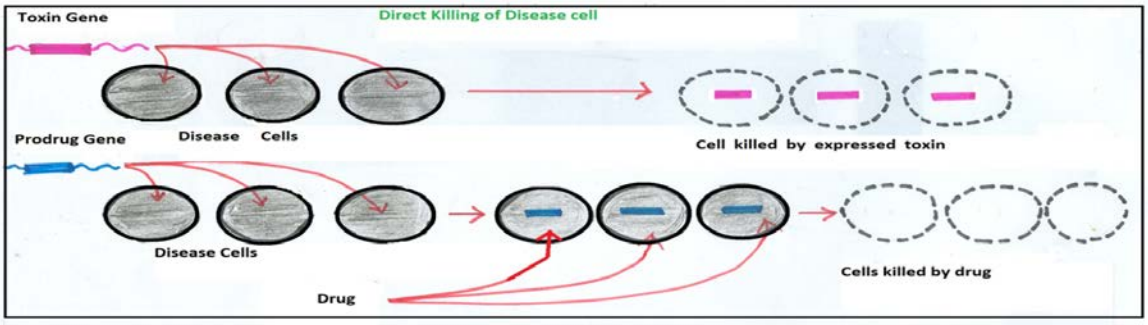

Figure 8-1.3.2: a) Direct killing of diseased cells by two methods. The first method is the introduction of toxin gene into the diseased cell which when expresses toxin protein the cells die. The second method involves incorporation of a certain gene (e.g. TK) in the gene therapy vector which shows a suicidal property on introducing certain drug (e.g. ganciclovir).

Thymidine kinase (TK) phosphorylates the introduced prodrug ganciclovir which is further phosphorylated by endokinases to form ganciclovir triphosphate, an competitive inhibitor of deoxyguanosine triphosphate. Ganciclovir triphosphate causes chain termination when incorporated into DNA.

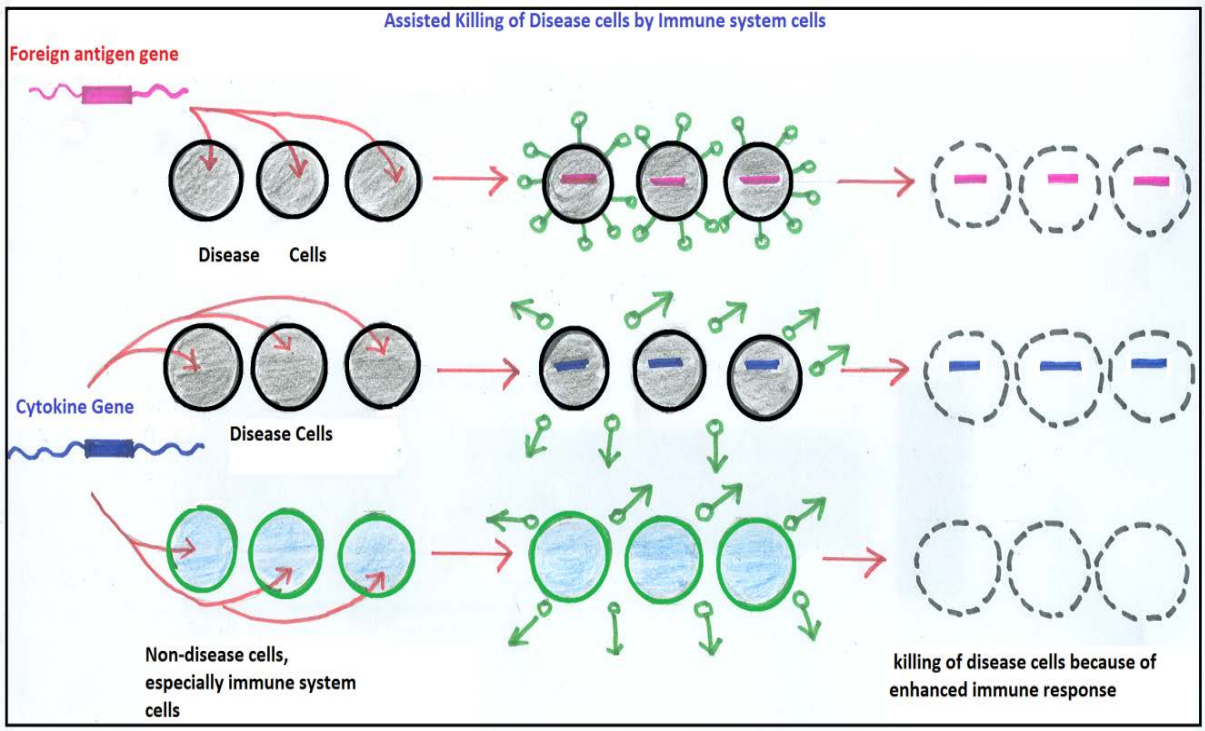

Figure 8-1.3.2: b) Assisted killing is another strategy of killing diseased cells. Here one method is to insert a well known foreign antigen coding gene which induces immune cells for the killing of the diseased cells. Few more methods are based on immune cells activation in which a certain cytokine encoding gene incorporated into gene therapy vector and inserted into either diseased cells or non-diseased cells. This will lead to enhanced immune response followed by killing of diseased cells.

8-1.3.3 Targeted Inhibition of Gene Expression

This is to block the expression of any diseased gene or a new gene expressing a protein which is harmful for a cell. This is particularly suitable for treating infectious diseases and some cancers.

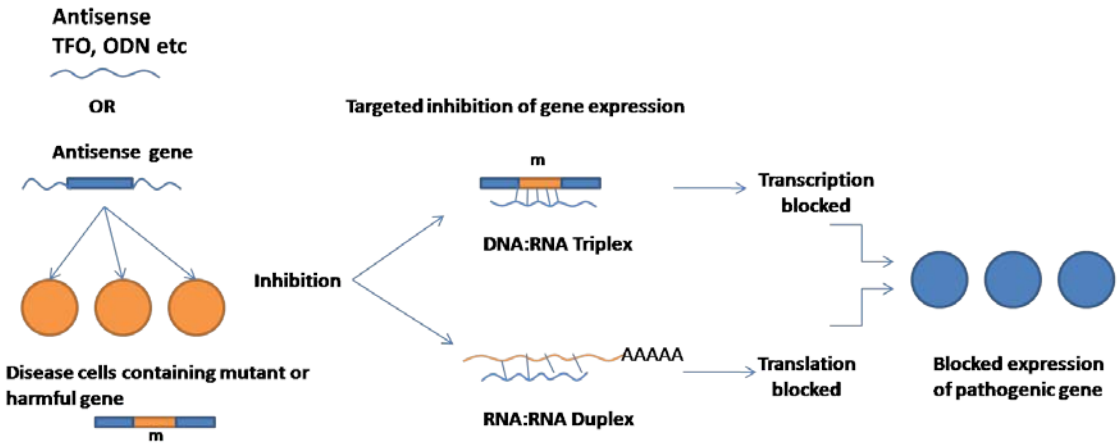

Figure 8-1.3.3 To inhibit the target gene expression in diseased cell the antisense mRNA coding gene inserted vector or triplex-forming oligonucleotides (TFO) or antisense oligonucleotide (ODN) can be introduced which will inhibit the gene expression either by forming DNA:RNA triplex inside the nucleus or forming RNA:RNA duplex by forming complementary mRNA strand of disease protein coding mRNA. This may lead to blocking of disease causing protein expression.

8-1.3.4 Targeted Gene Mutation Correction

It is used to correct a defective gene to restore its function which can be done at genetic level by homologous recombination or at mRNA level by using therapeutic ribozymes or therapeutic RNA editing.

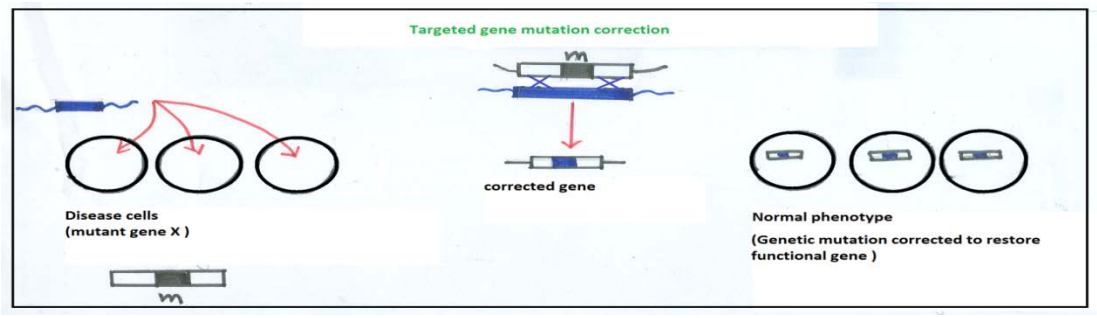

Figure 8-1.3.5 This is used for disease caused by mutation. The corrected gene will be swapped by the mutant gene X (m). Then diseased cells will become normal after the correction of mutation by gene therapy.

(Source: http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2871/?report=objectonly)

8-1.4 Gene Therapy Approaches

8-1.4 .1 Classical Gene Therapy

It involves therapeutic gene delivery and their optimum expression once inside the target cell. The foreign genes carry out following functions.

- Produce a product (protein) that the patient lacks;
- Produces toxin so that diseased cell is killed.
- Activate cells of the immune system so as to help in killing of diseased cells.

8-1.4 .2 Non-classical gene therapy

It involves the inhibition of expression of genes associated with the pathogenesis, or to correct a genetic defect and restore the normal gene expression.

8-1.5 Methods of gene therapy

There are mainly two approaches for the transfer of genes in gene therapy:

1**.** Transfer of genes into patient cells outside the body (*ex vivo gene therapy*)

2. Transfer of genes directly to cells inside the body (*in vivo*).

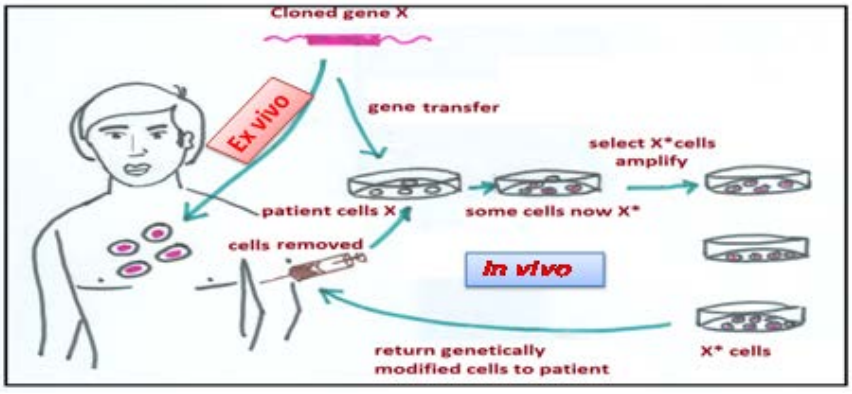

Figure 8-1.5 Gene Therapy using autologous cells: Cells are used, i.e. cells are removed from the patient, cultured *in vitro*, before being returned to the patient's body. In this figure *in vivo* and *ex vivo* gene therapy is diagrammatically explained.

(Source: *http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2897/?report=objectonly*)

8-1.5.1 *Ex vivo* gene therapy

- In this mode of gene therapy genes are transferred to the cells grown in culture, transformed cells are selected, multiplied and then introduced into the patient.
- The use of autologous cells avoids immune system rejection of the introduced cells.
- The cells are sourced initially from the patient to be treated and grown in culture before being reintroduced into the same individual.
- This approach can be applied to the tissues like hematopoietic cells and skin cells which can be removed from the body, genetically corrected outside the body and reintroduced into the patient body where they become engrafted and survive for a long period of time.
- Figure 8-1.5.1 shows a self explanatory schematic diagram for e*x vivo* gene transfer.

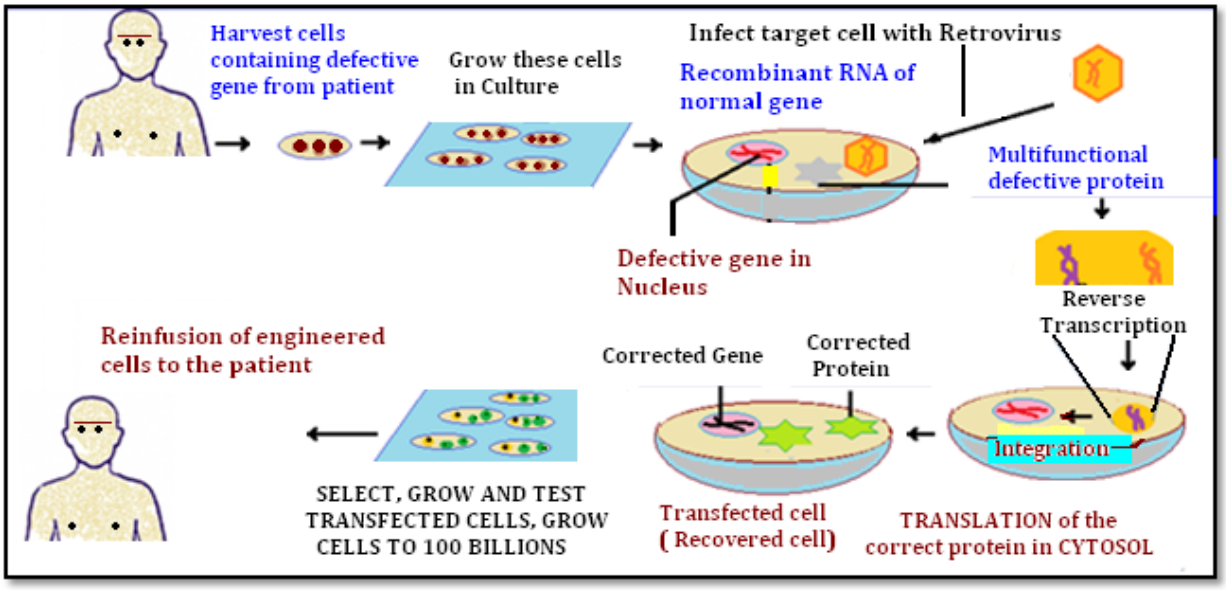

Figure 8-1.5.1 *Ex vivo* therapy involves tightly regulated cellular manipulation in harvested cells

8-1.5.2 *In Vivo* Gene Therapy

- *In vivo* method of gene transfer involves the transfer of cloned genes directly into the tissues of the patient.
- This is done in case of tissues whose individual cells cannot be cultured *in vitro* in sufficient numbers (like brain cells) and/or where re-implantation of the cultured cells in the patient is not efficient.
- Liposomes and certain viral vectors are employed for this purpose because of lack of any other mode of selection.
- In case of viral vectors such type of cultured cells were often used which have been infected with the recombinant retrovirus *in vitro* to produce modified viral vectors regularly. These cultured cells will be called as vector-producing cells (VPCs)). The VPCs transfer the gene to surrounding disease cells.
- The efficiency of gene transfer and expression determines the success of this approach, because of the lack of any way for selection and amplification of cells which take up and express the foreign gene.
- Figure 8-1.5.2 shows various steps of *in vivo* gene transfer.

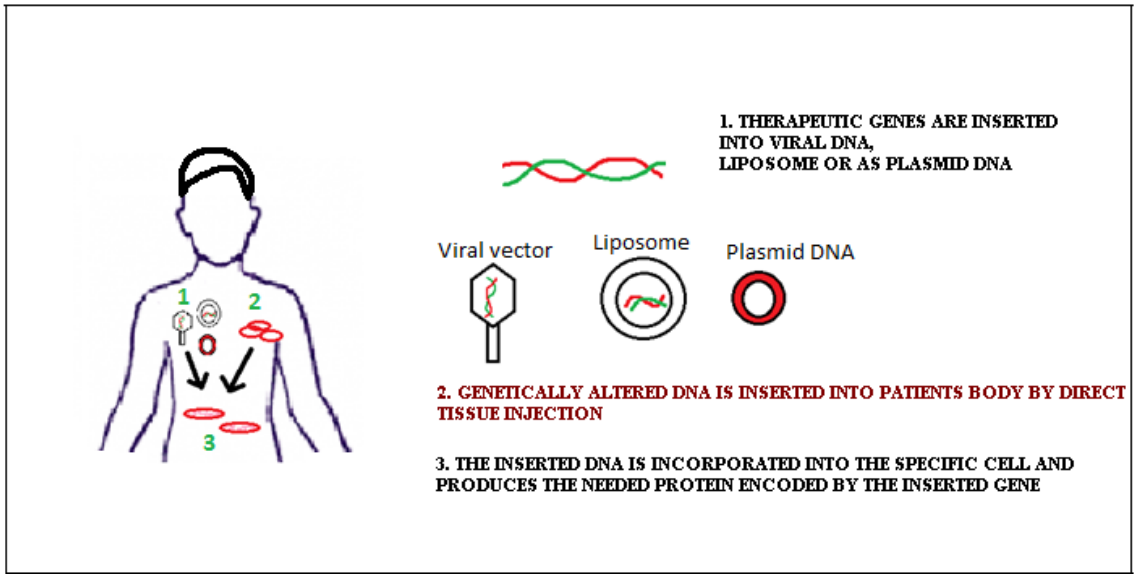

Figure 8-1.5.2 various steps of *in vivo* gene transfer

Table 8-1.5.1: Differences between In Vivo and Ex Vivo gene therapy

Difference Between *in vivo* and *ex vivo* Gene Delivery Systems	
In vivo	***Ex vivo***
Less invasive	More invasive
Technically simple	Technically complex
Vectors introduced directly	No vectors introduced directly
Safety check not possible	Safety check possible
Decreased control over target cells	Close control possible

8-1.6 Target sites for Gene Therapy

Therapeutic genes have to be delivered to specific target sites for a specific type of disease. This table describes the list of such disease and their target sites for gene therapy.

Table 8-1.6.1: Target cells for gene transfer

Target cells for gene transfer	
Disease	**Target Cells**
Cancer	Tumor cells, antigen presenting cells (APCs), blood progenitor cells, T cells, fibroblasts, muscle cells
Inherited monogenic disease	Lung epithelial cells, macrophages, T cells, blood progenitor cells, hepatocytes, muscle cells
Infectious disease	T cells, blood progenitor cells, antigen presenting cells (APCs), muscle cells
Cardiovascular disease	Endothelial cells, muscle cells
Rheumatoid arthiritis	Sinovial lining cells
Cubital tunnel Syndrome	Nerve cells

8-1.7 Vectors for gene therapy

Vectors for gene therapy can be classified into two types:

1. Viral vectors
2. Non-viral

Note: Table 2 shows vectors used in gene therapy. It is adapted From AR Prabhakar in *Gene Therapy and its Implications in Dentistry*. International Journal of Clinical Pediatric Dentistry, 2011; 4(2):85-92

Table 8-1.7: Vectors used in gene therapy

Vectors used in gene therapy	
Viral Vector	**Non-viral Vectors**
Adenovirus	Lipid complex
Retrovirus	Liposomes
Adeno- Associated Virus	Peptide/protien
Lentivirus	Polymers
Vaccinia virus	
Herpes simplex virus	
• Direct gene transfer methods like mechanical, electroporation, gene gun are also ued to transfer genes into target cells.	

8-1.7.1 Viral vectors

Retroviruses, adenoviruses and adeno-associated viruses (AAV) some commonly used viral vectors whereas some less commonly used viral vectors are derived from the Herpes simplex virus (HSV-1), the baculovirus etc.

- **Adenoviral vectors**

Adenoviruses are large linear double-stranded DNA viruses that are commonly used for preparing gene transfer vectors. Adenovirus vectors are known to be the second most popular gene delivery vector for gene therapy of various diseases like cystic fibrosis and certain types of cancer. Figure 8-1.7.1.1 shows how the adenoviruses enter cells by receptor-mediated endocytosis. A primary cellular receptor binds to viral fiber then the virus interacts with secondary receptors which are responsible for its internalization. Coxsackie and Adenovirus Receptor (CAR), Heparan sulphate glycosaminoglycans, sialic acid, CD46, CD80, CD86, alpha domain of MHC I are the primary receptors which are specific for specific strains of adenovirus. Integrins are the secondary receptors which helps in the internalization of viral particles. Some adenovirus directly interacts with integrins like in the case of fiber deficient Ad2 virions.

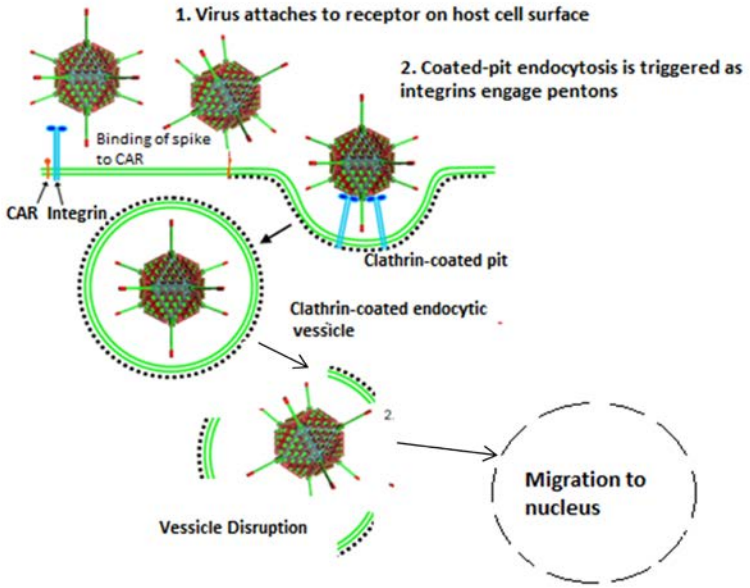

Figure 8-1.7.1.1a

***Adapted and modified from*: http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2918/?report=objectonly**

The adenoviral DNA has inverted terminal repeats (ITRs) and a terminal protein (TP) is attached covalently to 5' termini. The adenoviral genome is classified as early and late regions based on the proteins they express. Proteins encoded by early region (E1, E2, E3, E4) genes are involved in viral DNA replication, cell cycle modulation and defense system. The late region genes (L1, L2, L3, L4, L5) encodes the viral structural proteins. Three classes of adenoviral vectors namely first, second and third generation viral vectors are developed for gene therapy purpose.

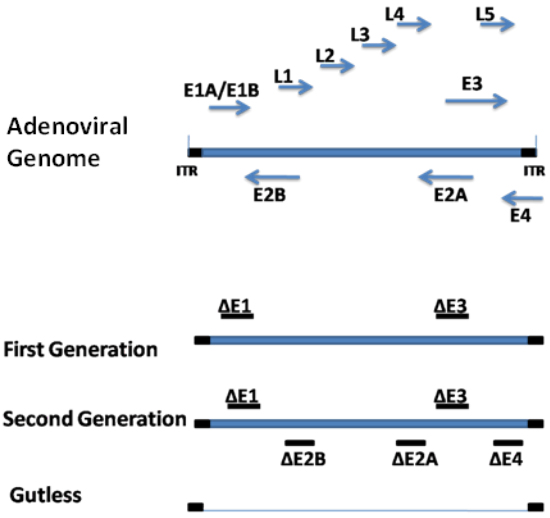

Figure 8-1.7.1.1b Map of Adenoviral genome and construction of different types of adenoviral vectors

Adapted and Modified from: R Alba, A Bosch and M Chillon (2005). Gutless adenovirus: last-generation adenovirus for gene therapy. Gene Therapy, 12, S18-S27

First generation adenoviral Vectors

These vectors are constructed by replacing the E1/E3 expression cassette and inserting our candidate gene of 3-4kb size. E1 encodes proteins responsible for expressions of other viral genes required for viral growth. So cell lines that can provide E1 proteins *in trans* are required for the replication of the E1 deleted viral vectors.

Advantages:

- They are human viruses produced at very high titers in culture.
- They can infect a wide range of human cell types including non- dividing cells.

- They enter into cells by receptor mediated endocytosis with a very high transduction efficiency reaching upto 100% *in vitro*
- Their large size enables them to accept large inserts.

Disadvantages:

- Expression of foreign gene is for short period of time as they do not integrate into the chromosome.
- These vectors may generate immune response causing chronic inflammation.

Second generation adenoviral Vectors

These vectors have been developed to overcome these difficulties. Here of E1/E2 or E3/E4 expression cassettes are called deleted and replaced. The E1/E2 or E3/E4 proteins are required for viral DNA replication. Similar to first generation viral vector, cell lines which can complement both E1and E2 or E3 and E4 are needed. It can carry DNA insert upto 10.5kb

Advantages:

- It has improved safety and increased transgene expression.

Disadvantages:

- These viral vectors are associated with immunological problems.
- Construction of these vectors is difficult.

Third generation adenoviral Vectors

These vectors are otherwise called as **gutless adenovirus.** These are also known as helper dependent adenovirus as they lack all the coding sequences and require helper virus which carries all the coding sequences. Helper virus for example AAV, or artificially disabled viruses provide the viral functions needed for successful infection like viral DNA replication, viral assembly and infection of new cells etc. The size of insert DNA can be 36kb and hence called as high

capacity adenoviruses. They carry only 5' inverted terminal repeats (ITR) and 3' packaging signals (ψ).

Advantages:

- These are non-integrative and high-capacity vectors.
- It can be produced in high titer and the construction of these vector is easy.
- It shows longer stability and reduced immune response.

Disadvantages:

- Helper virus contamination contamination can cause diseases like conjunctivitis, pharyngitis, cold and respiratory disease.

- **Adeno- Associated Virus (AAV)**

Adeno-associated viruses (AAVs) are a group of small, single-stranded DNA viruses which cannot usually undergo productive infection without co-infection by a helper virus, such as an adenovirus.

- The insert size for AAV is 4.5 kb, with the advantage of long-term gene expression as they integrate into chromosomal DNA.
- AAVs are highly safe as the recombinant adeno associated vectors contains only gene of interest and 96% viral genes are deleted.

Adeno-associated viruses are explained in detail in Module 5-Lecture 1.

- **Retroviral Vectors**

Retroviruses are RNA viruses which possess a reverse transcriptase activity, enabling them to synthesize a complementary DNA. Following infection (transduction), retroviruses deliver a nucleoprotein complex (pre-integration complex) into the cytoplasm of infected cells. The viral RNA genome is reverse transcribed first and then integrates into a single site of the chromosome.

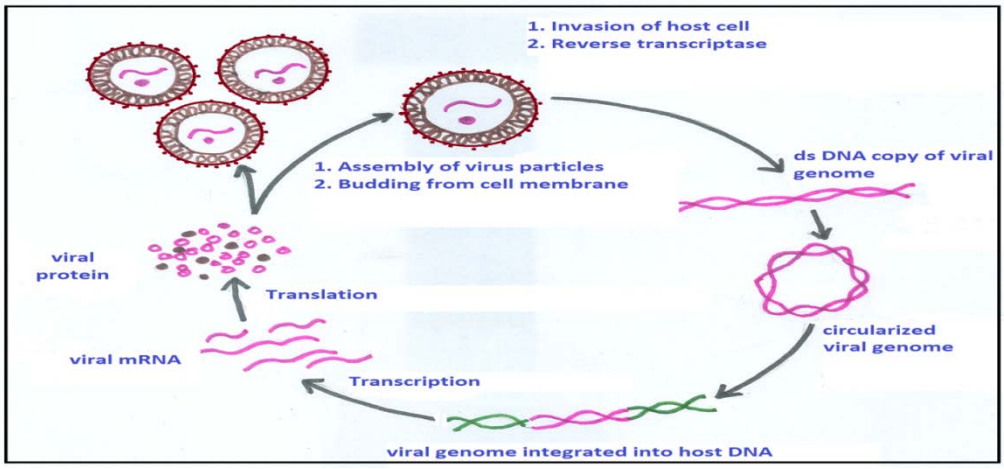

Figure 8-1.6.1.1c

Adaptedand modified from: http://www.ncbi.nlm.nih.gov/books/NBK7570/figure/A2357/?report=objectonly

- **Tumor retroviruses**, example Moloney's murine leukemia virus (MoMuLV), is widely used for the generation of recombinant vectors. these are produced at low titers as all the viral genes are deleted.
- **Oncoretroviruses:** The cells that divide shortly after infection can only be transduced by oncoretrovirus. The preinitiation complex is excluded and their entry is restricted in to the nucleus as they can only enter when nuclear membrane dissolves during cell division the target cells for this viral vector is limited

- **Recombinant lentiviruses** are being developed that are non- pathogenic to humans and have the ability to transduce stationary cells.

➢ **Other Viral Vectors:**

These include herpes simplex virus vectors and baculovirus.

Herpes simplex virus vectors: Herpes simplex virus-1 (HSV-1) is a 150 kb double-stranded DNA virus with a broad host range that can infect both dividing and non-dividing cells. the insert size is comparatively larger (>20kb) but have a disadvantage of short-term expression due to its inability to integrate into the host chromosome

Baculovirus: They can take up very large genes and express them highly efficiently. They help in recombinant protein expression in insect cell. They can infect hepatocytes as an only mammalian cell type and the gene expresses under the control of either mammalian or viral promoter.

Simian Virus 40 Vectors (SV40): SV40 are icosahedral papovavirus with a circular double stranded DNA of 5.2kb size as genetic material. The genome encodes for early proteins viz; large T antigen (Tag) and small t antigen (tag) and late protein viz; a regulatory protein agnoprotein and three structural proteins (VP1, VP2, VP3). The Tag gene is removed as it is responsible for inducing immunogenicity in the recombinant SV40 vector. All the structural proteins except the major capsid protein VP1 is removed resulting in a genome of 0.5kb size which includes origin of replication (ori) and encapsidation sequence. Recombinant SV40 vectors allows expression of transduced gene

8-1.7.2 Non- viral vectors

It involves chemical and physical methods such as direct injection of naked plasmid DNA (particle bombardment), receptor-mediated endocytosis and gene transfer through liposomes, polymers, nano particles etc.

Some non viral methods

- **Direct injection/particle bombardment:**

DNA can be injected parenterally which can be considered for Duchenne muscular dystrophy (DMD). An alternative approach uses particle bombardment (‘gene gun’) technique, in which DNA is coated on to metal micro particles and fired from a ballistic gun into cells/tissues. This technique is used to transfer the foreign DNA and its transient expression in mammalian cells *in vitro* and *in vivo* as well. It can cross the physical barriers like skin, muscle layer for which it is used for vaccination. Particle bombardment is used to deliver drugs, fluorescent dyes, antigenic proteins etc.

Advantage: Simple and comparatively safe.

Disadvantage:

- Poor efficiency of gene transfer.
- A low level of stable integration of the injected DNA. Repeated injection may cause damage in the proliferating cells.

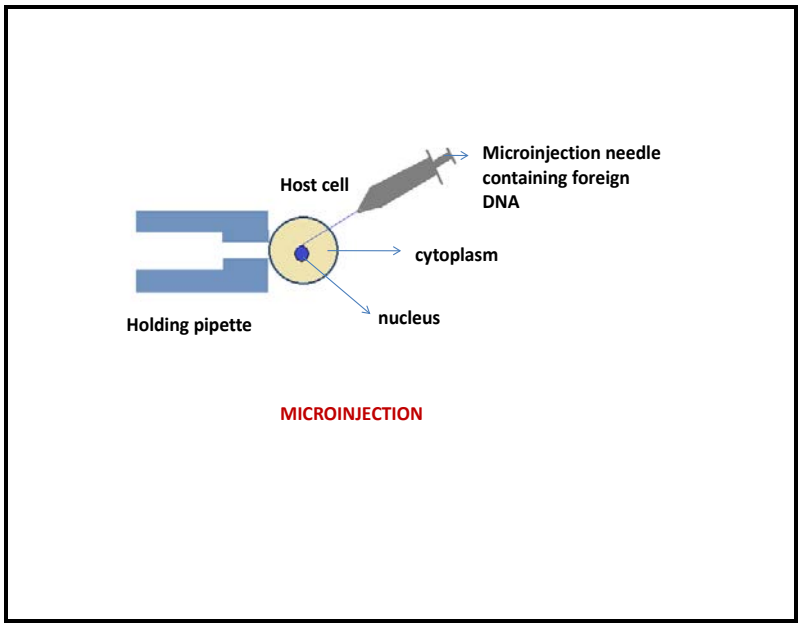

Figure 8-1.7.2.1 Microinjection process

Microinjection involves the delivery of foreign DNA, by the help of glass micropipette into a living cell. The cell is held against a solid support or holding pipette and micro neeedle containing the desired DNA is inserted into the cell. The tip of the pipette used is about 0.5 to 5 micro meter diameter which resembles an injection needle. For this, glass micropipette is heated until the glass becomes somewhat liquefied and is quickly stretched to ressemble a injection needle. The delivery of foreign DNA is done under a powerful microscope (micromanupulator).

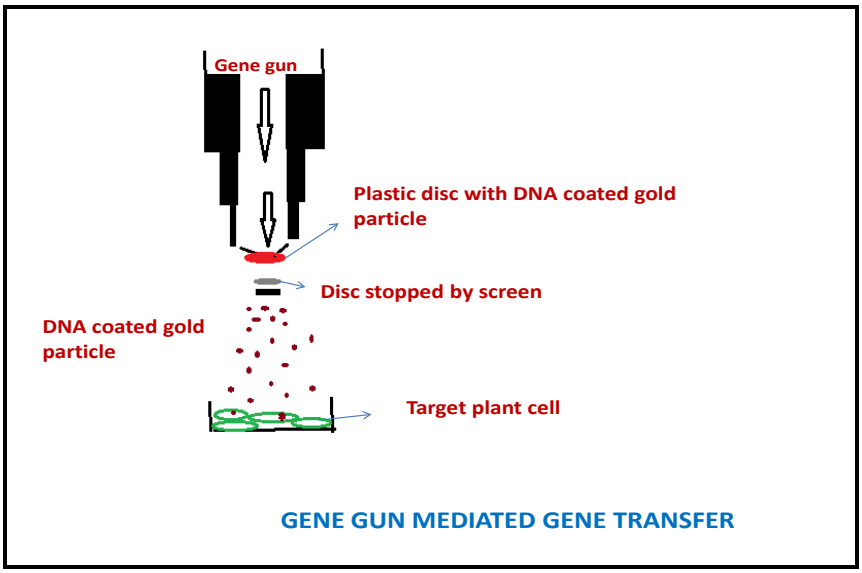

Figure 8-1.7.2.2 Gene gun mediated gene transfer

In **particle bombardment** method, the tungsten or gold particles (micro projectiles) are coated with the foreign DNA. Micro-projectile bombardment uses high-velocity metal particles to deliver biologically active DNA into the target cells. The macroprojectile is coated with the coated particles and is accelerated with air pressure and shot into plant the target tissue. A perforated plate is used, which allows the micro-projectiles to pass through to the cells on the other side of the plate and stops the macropojectile. Particle coated with the foreign gene releases the foreign gene when enters into the target cell and integrates into the chromosomal DNA. This technique is also used to transfer genes in mammalian cells. Mammalian cell lines like HEK 293, MCF7 showed gene expression when transfected with luciferase and green fluorescent genes and their gene expression was dependent on helium pressure, size and amount of gold particle and DNA load on each particle. Cell viability depends on helium pressure.

(See lecture 3 of module 5 for advantages and limitations)

- **Liposomes Mediated**

Liposomes are spherical vesicles which are made up of synthetic lipid bilayers which imitate the structure of biological membranes. DNA to be transferred is packaged into the liposome *in vitro* and transferred to to the targeted tissue. The lipid coating helps the DNA to survives *in vivo* and enters into the cell by endocytosis. Cationic liposomes,

where the positive charge on liposomes is stabilized by binding of negatively charged DNA, are popular vehicles for gene transfer *in vivo.* For more detail see lecture 2 of module 5.

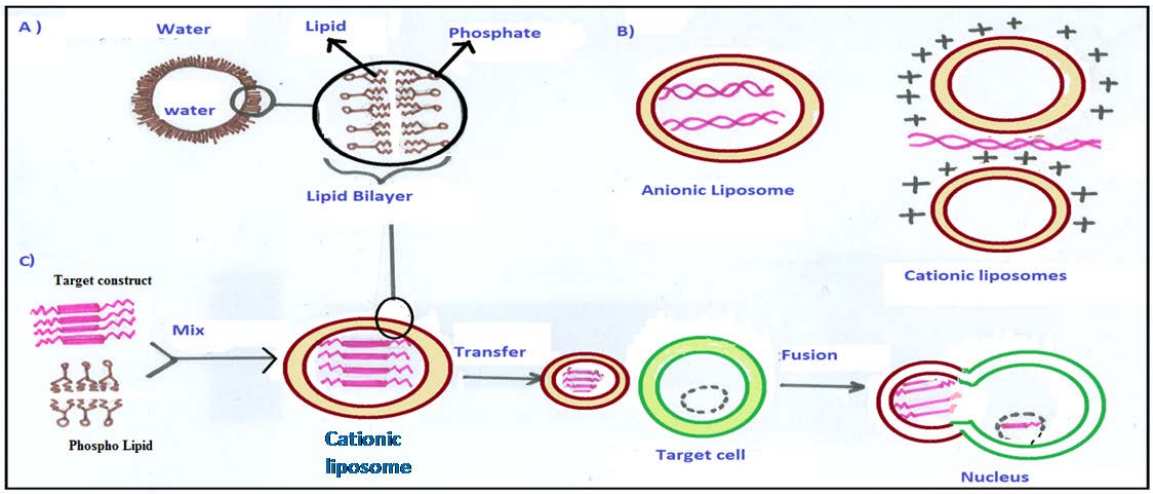

Figure 8-1.7.2.4: In vivo liposome mediated gene transfer- (A) formation of lipid bilayer in water (B) Structure of anionic and cationic liposome (C) Use of liposome to transfer genes into cells.

Adapted and modified from: http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2924/?report=objectonly

Advantage:

- The liposomes with the foreign DNA are easy to prepare.
- There is no restriction in the size of DNA that is to be transferred.

Disadvantage:

- Efficiency of gene transfer is low and transient expression of the foreign gene is obtained as they are not designed to integrate into the chromosomal DNA.

➢ **Electroporation**

In electroporation, the external electric field is applied to the protoplast, which changes the electrical conductivity and the permeability of cell membrane; and thus the exogenous molecules found in the medium are taken up to either the cytoplasm (transient transfection) or into the nucleus (stable transfection).The efficiency of electroporation can be increased by giving the cell a heat shock, prior to the application of electric field

or by using small quantity of PEG while doing electroporation. See lecture 3 of module 5 for detailed explanation.

Advantage:

- By electroporation large numbers of cells can be processed at once, and thus the amount of time spent processing cells can be cut down.

Disadvantages:

- If the voltage applied is not calculated properly, the cells may damage.
- If electroporation does not occur in controlled environment, the potentially harmful substances can enter the cell or the impurities from solution may enter. This is because there is no way to control what enters the cell membrane.

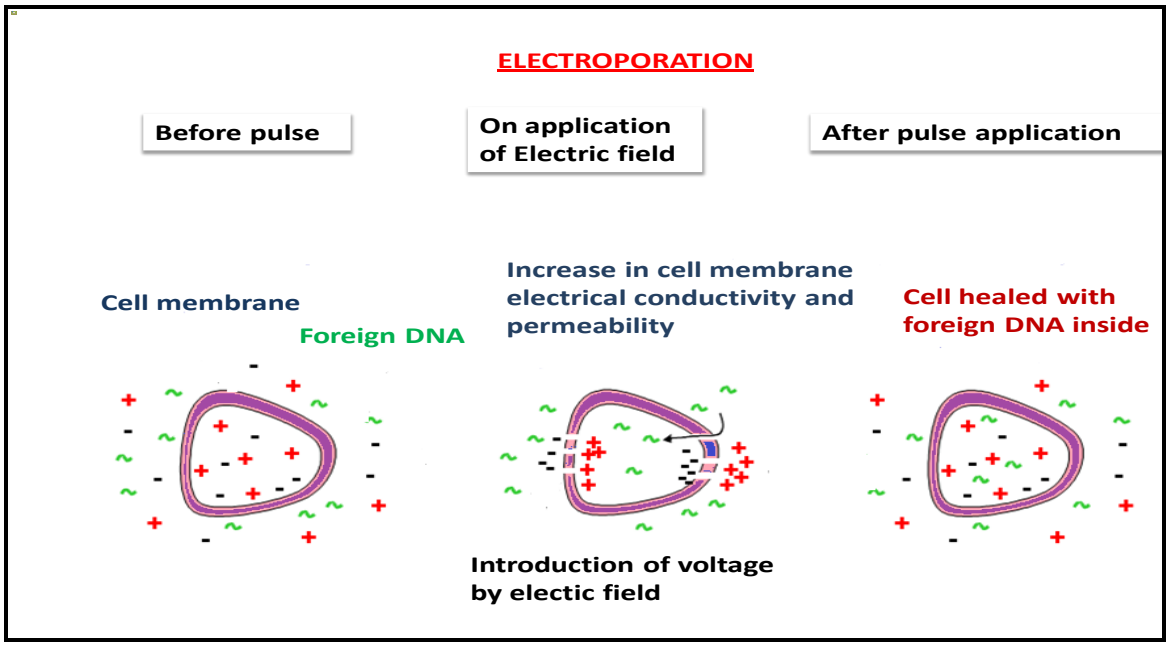

Figure 8-1.7.2.5 Electroporation

- **Sleeping Beauty Transposition**

This is a non viral method of gene transfer which offer the advantage of stable DNA integration into the chromosomes of vertebrates. The sleeping beauty transposition system consists of a sleeping beauty transposon and a sleeping beauty transposase. The SB transposon consists of two terminal inverted repeats present each at the ends of the gene of interest and the SB transposase mediates excision of the SB transposon and its integration into a site of chromosome having a dinucleotide dimer TA by cut and paste mechanism.

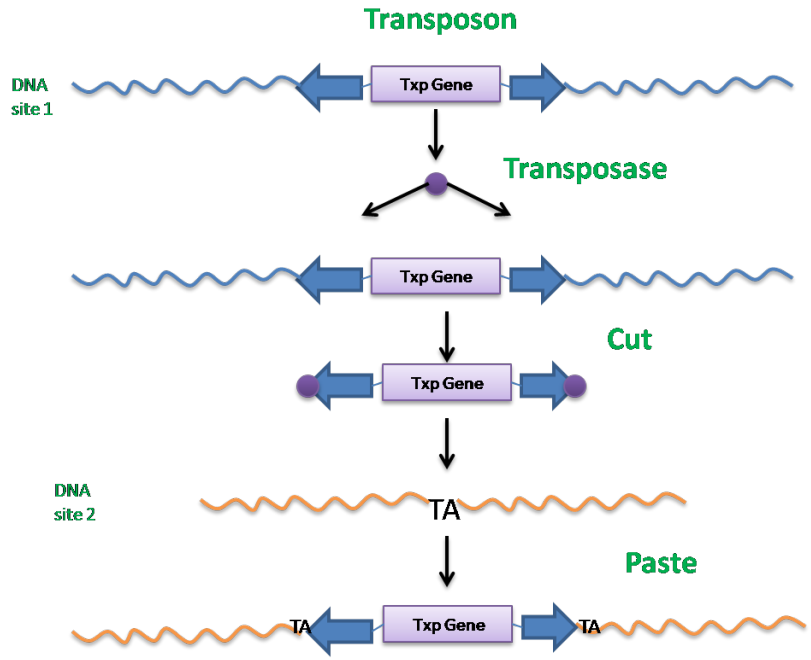

Figure 8-1.7.2.6 Cut and paste mechanism of a DNA transposon by a transposase (txp gene)

Adapted and modified from: **http://www.discoverygenomics.net/pdf/SBT_ProlongedExpressions.pdf**

For gene therapy transposase gene is replaced by our gene of interest and transposase is provided in trans as shown in Figure8-1.7.2.7.

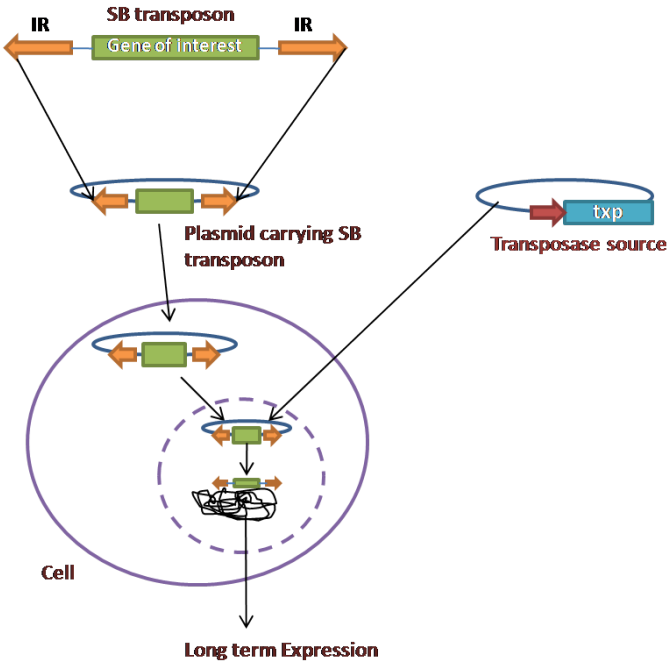

Figure8-1.7.2.7 Mechanism of gene transfer by sleeping beauty transposition

Adapted and modified from: **http://www.discoverygenomics.net/pdf/SBT_ProlongedExpressions.pdf**

- **RNA-DNA Chimera**

This RNA- DNA chimera also known as chimeroplast is used to correct point mutations by mismatch repair. This 68 nucleotides long double stranded nucleic acid molecule comprises of one strand DNA and another strand consisting of two 10 nucleotides long 2'-O methyl RNA stretch separated by a 5 nucleotide long DNA stretch as shown in Figure 8-1.7.2.8. The pentameric chimeric DNA carries the mismatch and the other DNA strand has its complementary bases. Additionally this RNA-DNA chimera consists of two hairpin loop and a GC clamp.

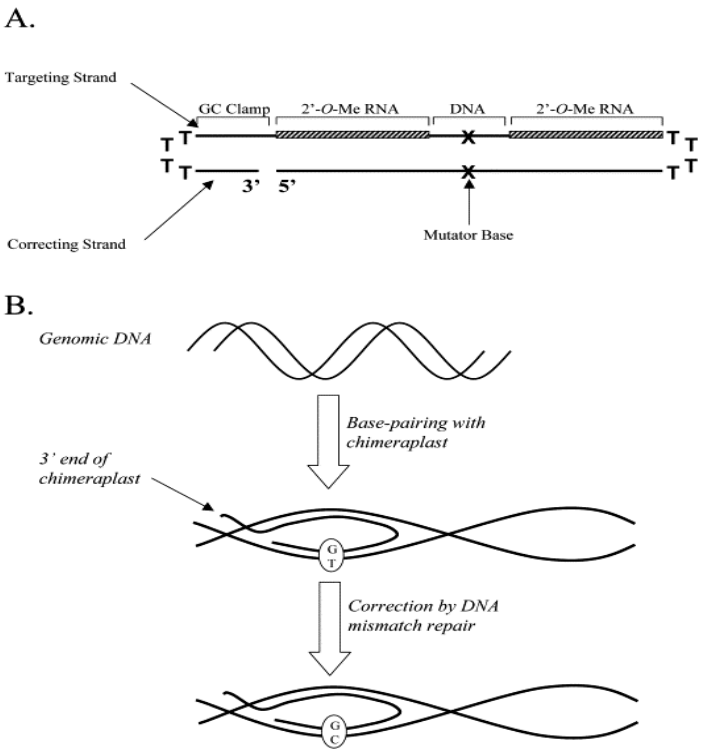

Figure 8-1.7.2.8: Structure and mechanism of action of RNA DNA chimera

Source: **http://www.sciencedirect.com/science/article/pii/S0925443902000686**

- **Receptor-Mediated Endocytosis**

It can be both viral and non-viral mediated gene transfer. Viral vectors attach to the surface receptors through viral surface components and internalized. In non viral mode of receptor mediated endocytosis DNA is first coupled to a ligand that binds specifically to a cell surface receptor and causes transfer of the DNA into cells by endocytosis. Coupling is done by linking the receptor molecule with polylysine followed by reversible binding of the negatively charged DNA to the positively charged polylysine component. Transferrin receptor which is comparatively abundant in proliferating cells and hematopoietic cells is utilized as a target and transferin as a ligand in this approach.

Advantage: Gene transfer efficiency may be high.

Disadvantage:

- It does not allow integration of the transferred genes. Also, the protein— DNA complexes are not stable in serum.

Coupling of inactivated adenovirus to the DNA-transferin complex can increase gene transfer efficiency which help in receptor mediated endocytosis and lysosomal escape.

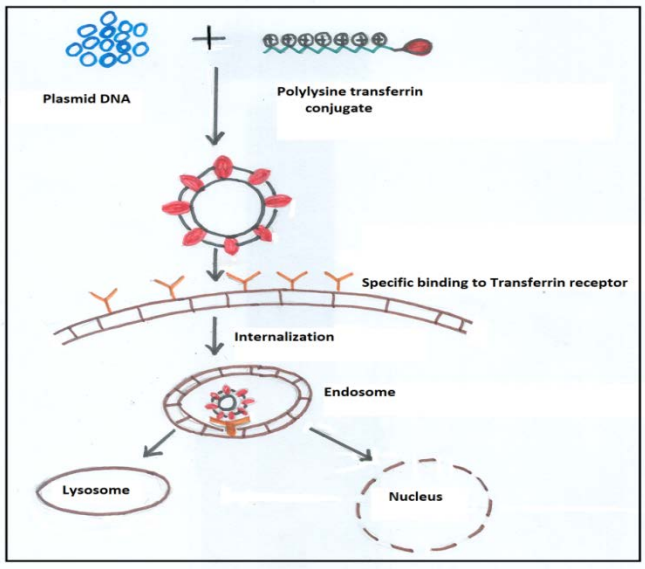

Figure 8-1.7.2.3 Receptor-Mediated Endocytosis

Adapted and modified from: http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2927/?report=objectonly

8-1.8 Endosomal/Lysosomal Escape

In gene therapy the gene becomes entrapped in the endocytic pathway and is degraded by the hydrolytic enzymes which are a major limiting step in gene therapy. There are various approaches to escape this endocytic pathway for both viral and non-viral gene delivery systems.

- **For viral gene delivery system**

  Enveloped virus can penetrate through endosome membrane and non enveloped viruses escape by forming pores or lyse the vesicular membrane.

- **For non-viral gene delivery system**

There are various strategies developed non-viral vector system for endosomal escape.

- **The 'proton sponge' hypothesis**: 'Proton sponge' effect has been seen in some cationic polymers which contain protonable amine groups with pKa close to endosomal/lysosomal pH can be used for gene delivery During acidification of endosomes the cationic polymers become protonated so that the H^+ influx is increased and thereby Cl^- concentration is increased inside the endosome leading to water influx. As a result the endosome swells and eventually ruptures.

- **Flip-Flop mechanism:** Due to electrostatic interaction of cationic lipoplexes and anionic lipids of endosomes facing towards cytoplasm there is lateral diffusion of lipids resulting in displacement of nucleic acids from lipoplexes to cytoplasm.

- **Pore formation**: In a peptide based gene delivery system a peptide named GALA which can undergo conformation change at low pH forms a pore in the vesicular membrane when incorporated.

- **Photochemical internalizaton**: This technique uses photosensitizers which can bind to and localize in the plasma membrane. These photosensitizers become confined to endosomal membrane during

endocytosis and remain inactive. When irradiated with light of a particular wavelength they produce reactive oxygen species resulting in lysis of endosomes.

8-1.9 Advantages of Gene Therapy

- Gene therapy can cure genetic diseases by addition of gene or by removal of gene or by replacing a mutated gene with corrected gene.
- Gene therapy can be used for cancer treatment to kill the cancerous cells.
- Gene expression can be controlled.
- Therapeutic protein is continuously produced inside the body which also reduces the cost of treatment in long term.

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MODULE 8: LECTURE 2

GENE TARGETING & SILENCING

8-2.1 Introduction

In previous lecture we learned about basic concept and methods of gene therapy we also know that with the help of gene therapy we can cure many genetic diseases caused by activation or deactivation of a gene due to some mutation in the genome. Gene therapy includes basically two strategies to cure the genetic defects (1) gene targeting or (2) gene silencing. Gene targeting as well as silencing is a technique which involves modification of the structure of a specific gene in the chromosome of a living cell in order to rectify the defective gene. A modified gene fragment can be replaced by the endogenous wild type gene and the phenotypic alteration can be assessed in the organism. The process involves the cloning of a piece of DNA in a gene targeting vector, which is then introduced into the cell where it replaces or modifies the abnormal gene in the chromosome through the process of homologous recombination. It can be used for deleting a gene, removing exons, adding a gene, and introducing point mutations. Briefly, gene targeting involves gene augmentation in organisms which can be permanent or conditional where as gene silencing is knock-out of the gene to cure the disease. Martin Evans, Mario Capecchi and Oliver Smithies won Nobel Prize of 2007 in Medicine or Physiology for their work on genetic modification using stem cells

8-2.2 Gene Target Construct

The gene targeting construct is usually a plasmid in which two long stretches of genomic DNA sequences are attached. These sequences are homologous to target site and known as homology arms. The homology arms direct the homologous recombination which finally results in the insertion of the construct in the host genome. BAC (Bacterial artificial chromosomes) can also be used as targeting vectors. In targeting vectors a selectable marker should be present which enables

selection of transfected cells and increases the targeted recombination products. Two types of selectable markers are used.

- **Positive selection marker**: It is used to isolate rare stably transfected cells. It is inserted within the homologous gene in the vector to make it non-functional and used as mutagen. (E.g.: *neo* gene for Neomycin resistance).

- **Negative selection marker**: It eliminates random insertions and insertion of heterologous components. (E.g.: TK gene for Thymidine kinase enzyme).

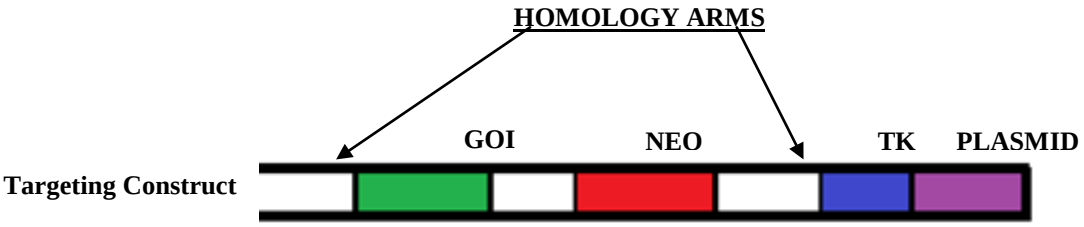


Figure 8-2.2: A targeting construct contain gene of interest (GOI) with two flanking homology arms on both sides. The positive selection marker neomycin resistance (*neo*) gene in between GOI and the end homology arm. This cassette will be used for swapping the GOI. The negative selection marker (i.e. TK gene) should be outside of the cassette which be used for detection of failure of experiment.

8-2.3 Gene Targeting

So far we have discussed about basics of gene targeting and gene silencing. Now in this section we are going to discuss following sub-topics related to gene targeting:

- Gene Targeting In Embryonic Stem (ES) Cells
- Homologous Recombination
- Positive Negative Selection (PNS) Strategy

8-2.3.1 positive and negative screening

The inner cell mass of the developing blastocyst are the source of ES cells. A targeting construct is introduced into ES cells by electroporation. These cells are then subjected to drug selection to enrich for homologous recombinant clones of the ES cells. Figure 8-2.3.1 shows generation of pure population of recombinant ES cells for gene therapy.

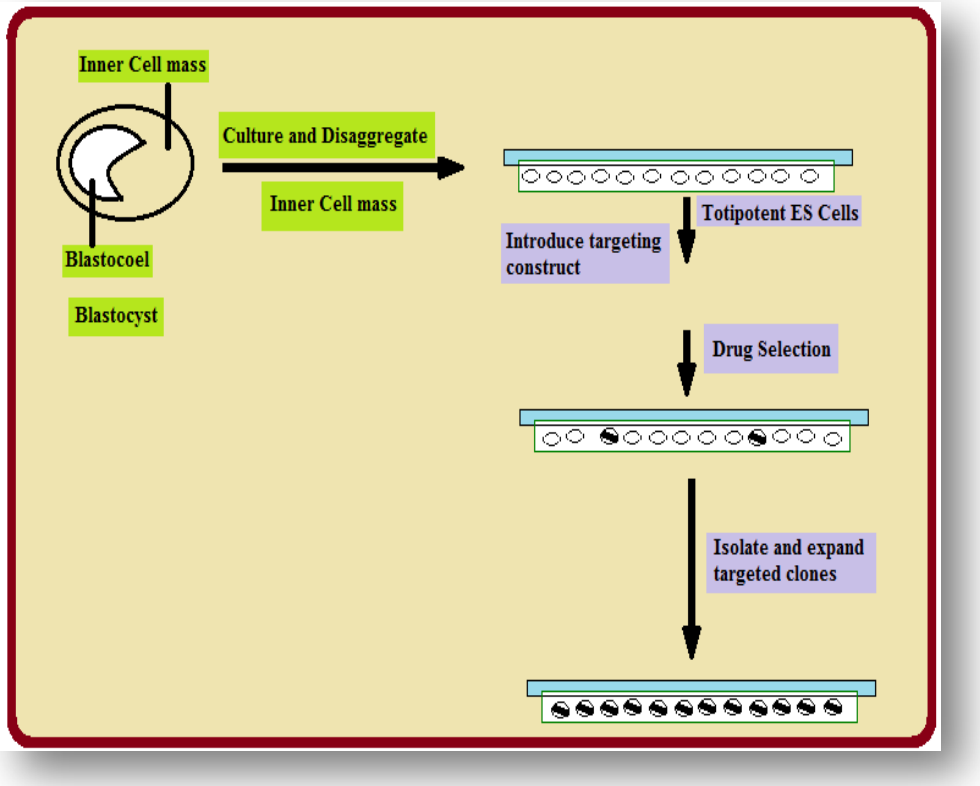


Figure 8-2.3.1: The inner cells from developing embryo of diseased subject were taken and developed a totipotent embryonic stem cell line. The target construct for the disease was introduced to the cell line. After successful gene transfer the positive and negative screening will be done. The pure positive clones were isolated and grown separately for therapeutic purpose.

8-2.3.2 Homologous Recombination

Homologous recombination is the natural recombination phenomenon by which nucleotide sequences are exchanged between two sister chromatids in cell division. This natural ability of the cell is utilized to shuffle the target construct to the genomic DNA. The targeting construct should be prepared according to the above described strategies in which the gene of interest must be carried by flanking homology arms and both positive and negative selection markers (i.e. *neo* gene and TK gene respectively). The target construct gets incorporated into the target location after successful homologous recombination. Figure 8-2.3.2 explains the homologous recombination steps in order to generate gene therapy target construct.

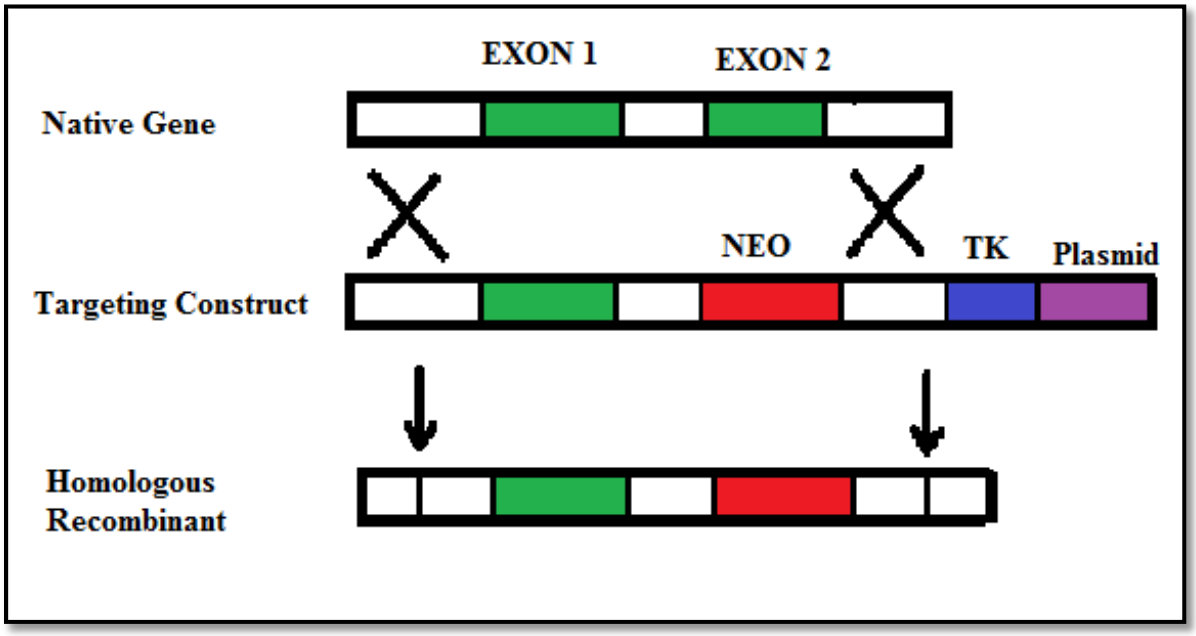


Figure 8-2.3.2: Homologous recombination between native gene (defective gene) and the target construct. The homology arms promote the homologous recombinant formation.

Adapted and modified from:

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/TransgenicAnimals.html

8-2.3.3 Positive Negative Selection (PNS) Strategy

The positive- negative selection of clone depends on the two marker genes incorporated in the target construct in which *neo* gene works for positive selection and TK gene works for negative selection that we have discussed earlier in this lecture. Here, when the cells were treated with target construct to perform natural homologous recombination the well incorporated positive clone has to be selected. For this the cells will be grown in a media which contain neomycin drug for positive selection and ganciclovir for negative selection. In presence of neomycin only those cells will grow which is incorporated with *neo* gene in their genome. In some cases there is a chance of wrongly incorporation of the gene. These types of cells can also grow in the presence of neomycin. Then another drug ganciclovir play a role to select further right clones. The ganciclovir is known as a synthetic analogue of 2-deoxyguanosine. This compound gets incorporated in to the DNA of dGTP by DNA polymerase which results in the termination of DNA elongation if the clone shows the activity of thymidine kinase (TK). Finally that population will be killed due to ganciclovir activity. Now the cells remained in the flask will be considered as pure positive clones. These cells can be grown further and now can be screened for homologous recombination of the GOI by Southern blot analysis or PCR. After screening, the obtained homologous recombinant clones are now used to propagate for therapeutic purpose.

8-2.4 Applications of Gene Targeting

Some of the applications of gene targeting are:

1. Mapping of gene expression with the help of marker genes such as lacZ gene.
2. Sophisticated genetic alterations
3. Conditional Gene Targeting
4. Null Mutations in vitro
5. Development of Disease Models

8-2.5 Gene Silencing

Gene silencing is a term of gene regulation used to describe the "switching off" of a gene by a mechanism without introducing any genetic modification. DNA is transcribed into mRNA but the mRNA is never translated into proteins. Gene regulation can be done either at the transcriptional level or at post-transcriptional level. In case of transcriptional level it is done by inducing modification in histone protein, changing the environment for the binding of transcriptional machineries such as RNA polymerase, transcription factors, etc. However in case of post-transcriptional level of gene regulations the transcribed mRNA by a particular gene is being blocked or destroyed. The post-transcriptional level of gene silencing is achieved by

1. Antisense Technology

2. RNA interference (RNAi)

8-2.5.1 Antisense Technology

Antisense technology talks about the production of complementary nucleic acid molecules against the mRNA molecule transcribed from the DNA in order to stop the translation into protein. These complementary molecules can be synthetically produced and delivered inside the cell to block the expression of diseased protein. It can be a short length of either RNA or DNA which commonly termed as Antisense oligonucleotides (AON). Here antisense refers the complementary nature of the synthetic molecule with respect to mRNA. When these AON inserted inside the cell it forms RNA duplex (i.e. double stranded RNA or RNA-DNA duplex). The formation of double stranded RNA inhibits gene expression at translation level as protein synthesis requires single stranded mRNA molecule as a template. This phenomenon is still not well understood but the current hypothesis about this is following-:

- blocking RNA splicing,
- accelerate the degradation process of the RNA and it also prevents the introns from splicing
- preventing the migration of mRNA from nucleolus to cytoplasm

- stopping the translation of diseased protein, and
- If complementary DNA molecule is used there may be a formation of triplex in DNA template.

➢ **Mechanism of Antisense Technology**

- The synthetic AON introduced inside the cell according to the gene of interest.
- If it is a DNA molecule it binds with the DNA inside the nucleus to form a triplex which inhibits the transcription and finally translation. Sometimes RNA-DNA heterodimer is also formed to stop the translation.
- In case of antisense RNA it binds with mRNA to stop the translation.
- Figure 8-2.4.1.1 shows the mechanism of antisense technology.

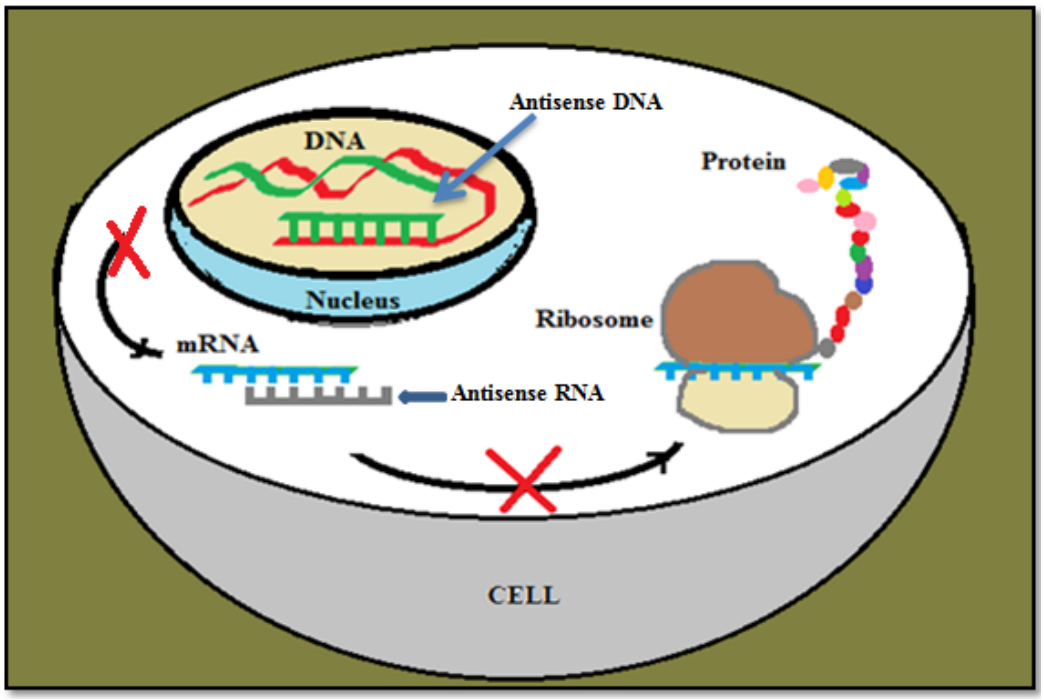


Figure 8-2.4.1 Mechanism of antisense technology: If antisense DNA is introduced into a diseased cell for the defective gene then it will bind the gene and form a triplex (dsDNA : ssDNA). This will stop the transcription of that gene and inhibit the protein synthesis. In another approach if the antisense RNA is designed for the mRNA then it will bind to the mRNA to form RNA duplex (RNA:RNA) and finally block the translation and degraded by dsRNA degradation pathway.

Adapted and modified from: http://www.scq.ubc.ca/antisense-rna/

- **Application of Antisense Technology**
  - In oncology antisense RNA has been used to inhibit many target proteins, such as growth factors receptors, growth factors, proteins responsible for invasion of cancerous cells and cell cycle proteins (Weiss *et al*, 1999).
  - If it is complementary to viral RNAs then may help in controlling various types of viral infections.
  - Development of animal models for long-term normal blood pressure in hypertensive animals (Richard Re *et al* 2000).
  - In January 2013 a drug called mipomersen (trade name Kynamro) got approval from the FDA for curing homozygous hypercholesterolemia.
  - Fomivirsen, an antiviral drug developed for the treatment of cytomegalovirus retinitis is basically an antisense oligonucleotide.

8-2.4.2 RNA interference

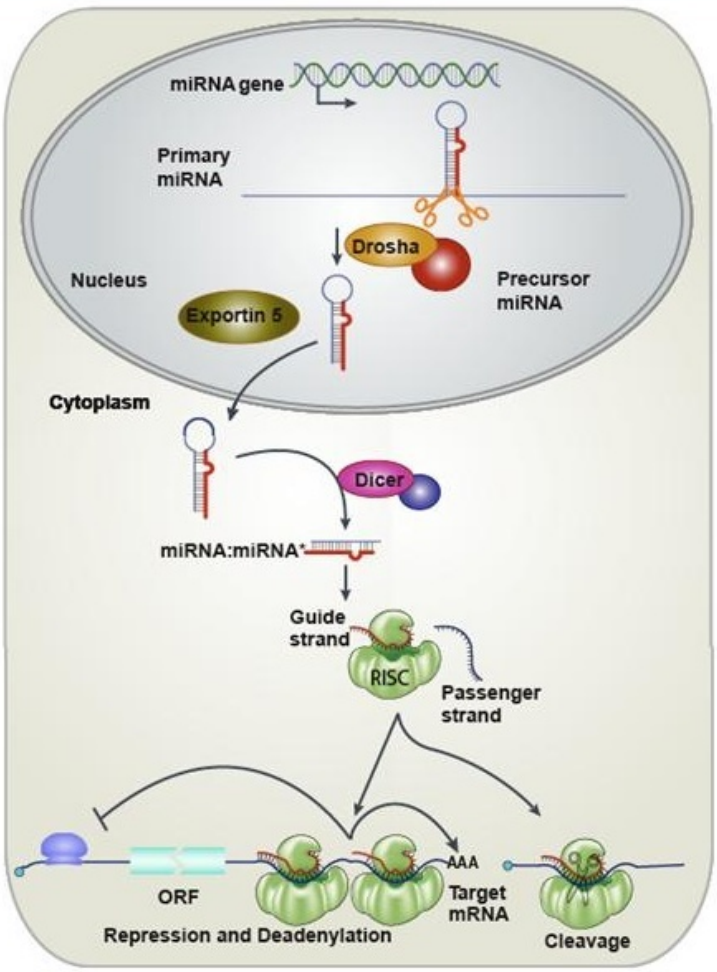
RNAi has shown its importance in the analysis of gene functions and silencing of gene for therapeutic purpose. It was first reported by Andrew Fire and his team in the year of 1997 while studying the introduction of dsRNA into *C. elegans* for silencing a gene unc-22 gene. RNA interference (RNAi) is basically a post-transcriptional phenomenon which may be triggered by providing a double-stranded RNA (dsRNA) which is known as double RNA activation. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) plays a central role in RNA interference based gene silencing. RNAi looks very similar to plant posttranscriptional gene-silencing (i.e.PTGS) and quelling in case of fungi. Functions of RNAi are as follows:

- Immunity : the immune response to viruses and other foreign genetic material
  (In case of plants)
- Down regulation of genes through mi RNA (micro RNA)
- Up-regulation of genes by using both siRNA and miRNA complementary to parts of a promoter

8-2.4.2.1 Micro RNA(miRNA) and Small Interfering RNA (siRNA)

Both are considered as interfering RNA. Historically miRNA was discovered in 1993 by Ambros and his coworkers where as siRNAs concept came in 1999 from another discovery in which a dsRNA showed its role in post-transcriptional gene silencing (PTGS) in plants by David Baulcombe's group. During PTGS at one stage there is a role of $\sim 20 - 25$ nt RNAs in silencing which was produced by the dsRNA. miRNAs is considered as regulators of cellular self genes(i.e. endogenous genes), and siRNAs act as guards of foreign or invasive genes coming from viruses, transposons, and transgenes etc. which try to get integrated into host genome.

- **Biogenesis of miRNAs and Role in Protein Regulation**



(Source: http://www.yale.edu/giraldezlab/miRNA.html)

Figure 8-2.4.2.1 Biogenesis of miRNAs and Role in Protein Regulation

The miRNA gene is always present in the host gnome which gets transcribed into primary-miRNA (pri-miRNA) first with the help of RNA polymerase II. This pri-miRNA is cleaved by an enzyme called Drosha which is a type of ribonuclease III enzyme. It liberates approximately 60 to 70 nt looped structure which is consider as precursor miRNA or pre- miRNA. This pre-miRNA is the transported with the help of Exportin 5 present in cytoplasm. Once the pre-miRNA is exported into cytoplasmic space the another dsRNA specific enzyme called Dicer helps in duplexing with other miRNA. The unwinding of the duplexed miRNA is done by helicase. Now the both dsRNA-specific endonucleases enzymes (Drosha and Dicer) help to generate 2-nucleotide-long-3' overhangs near the cleavage site. After unwinding of the double stranded miRNA the generation of target specific Guide strand and the passenger stand. Now the miRNA (i.e. Guide strand) is considered as mature miRNA which is then incorporated with the RNA-induced silencing complex (RISC). The target specific miRNA now bind with the mRNA and stop the translation. Finally the gene is silenced with the help of miRNA and the cell undergo self destruction pathway.

- **miRNA versus siRNA**

Table 8-2.4.2 A comparison between miRNA and siRNA

	Occurrence	**Configuration**	**Length**	**Complementary to target mRNA**	**Biogenesis**	**Action**	**Function**	**Clinical uses**
Micro RNA (miRNA)	Occur naturally in plants and animals	Single stranded	19-25 nt	Not exact, and therefore a single miRNA may target up to hun-dreds of mRNAs	Expressed by genes whose pur-pose is to make miRNAs, but they regulate genes (mRNAs) other	Inhibit transla te-ion of mRNA	Regulators (inhibitors) of genes (mRNAs)	Possible thera-peutic uses either as drug targets or as drug agents themselves. Expressi

					than the ones that expressed them			on levels of miRNAs can be used as potential diagnostic and biomarker tools
Short interfering RNA (siRNA)	Occur naturally in plants and lower animals. Whether or not they occur naturally in mammals is an unsettled question	Double stranded	21-22 nt	100% perfect match, and therefore siRNAs knock down specific genes, with minor off-target exceptions	Regulate the same genes that express them	Cleave mRNA	Act as gene-silencing guard-ians in plants and animals that do not have antibody-or cell-mediated immunity	siRNAs are valuable laboratory tools used in nearly every molecular biology laboratory to knock down genes. Several siRNAs are in clinical trials as possible therapeutic agents

(**Source:** George S. (2007). Mack MicroRNA gets down to business. *Nat. Biotechn.* 25: 631-638.)

- **Mechanism of RNAi based Gene Silencing**

  A plasmid vector along with the target construct has to be delivered inside the diseased cell. This vector is able to transcribe a double stranded shRNA (short hairpin RNA). This shRNA is first processed into siRNA (small interfering RNA) and then siRNA inhibit the mRNA translation by sequence specific degradation process thereby silencing the gene. In first step of formation of siRNA the shRNA bind to a ribonuclease enzyme (similar to RNase III) and cleaved into 21 to 25 nucleotide siRNA. These siRNAs are complexed with RNA Interference Specificity Complex (RISC). RISC helps siRNA to find the mRNA complementary sequence and formation of the duplex. The whole mechanism is well explained in figure 8-2.4.2.

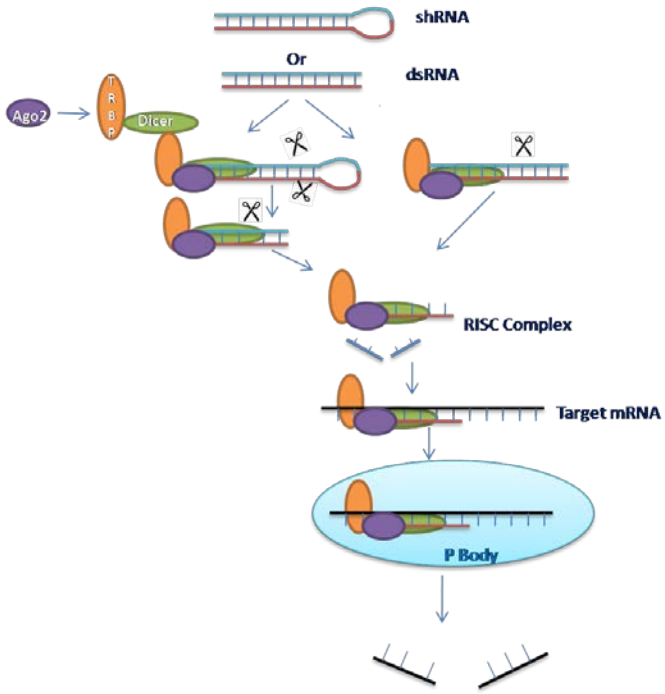


Figure 8-2.4.2.2: Steps involved in RNAi-mediated gene silencing

(*Adapted and modified from: http://www.invivogen.com/review-rna-interference*)

Steps involved in RNAi-mediated gene silencing in mammals using shRNAs-

1. The introduced plasmid has expressed a short hairpin RNA (shRNA). It requires Exportin 5 for the nuclear export.
2. Transactivating response (TAR) RNA-binding protein (TRBP) complexes with Dicer to form a dimer and then attaches to the shRNA.
3. Dicer generates 19-23 nucleotides siRNA and 2 nucleotides with 3' overhangs in one step only from the shRNA attached to the complex
4. Argonaute 2 (Ago 2) is a RNase which belongs to AGO subfamily and binds to the 3' overhang of siRNA in the RNA silencing complex resulting in unwinding of the dsRNA.,
5. The strand to which Ago 2 binds is called the guide strand and the other strand is known as "passenger strand". The later is cleaved by Ago2.
6. Now the "passenger strand" becomes free to leave the complex.
7. The integrated "guide strand" is now known as the active RISC (RNA Interference Specificity Complex). The RISC that contains various other argonautes and also few argonaute-associated proteins.
8. The siRNA sequence remained in the complex (i.e. guide strand) help the RISC to find the mRNA and bind at the correct location.
9. Now the RISC bring the mRNA to a processing bodies (i.e. P- bodies or GW-bodies). It is a cytoplasmic foci where mRNA decay factors are in high concentration which leads to the mRNA degradation.
10. The mRNA in the P-body is now cleaved by Argonaute 2 and then degraded.

- **Application of RNAi**

  1. Tool for studying gene expression and regulations
  2. **Medical applications:** Treatment of viral infections, cardiovascular diseases, cancer and metabolic disorders.

  Examples:

  – Treatment of age-related macular degeneration (AMD)
  – RNAi is used to block production of VEGF (Vascular endothelial growth factor).
  – Treatment of Hypercholesterolemia

- To block the production of LDL particles.

- **Advantage of RNAi**
  - It is target specific
  - Very small amount of small dsRNA is sufficient for silencing gene expression
  - A natural method of gene regulation
- **Limitations of RNAi**
  - Less is known regarding machinery and mechanism of RNA.
  - Uses of siRNA to therapeutic purposes is now more concerns about the subject safety as there may be a chance of disturbances in natural regulation of the immune system.
  - Problem of-target effects is not clear at present
  - Not much is known regarding Dosage requirement, stabilization & synthesis of tailored/engineered mRNA

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MODULE 8: LECTURE 3

GENE THERAPY IN THE TREATMENT OF DISEASES

8-3.1 Introduction

Gene therapy offers great promise as an alternative treatment method that can correct diseases caused by genes (hereditary) or environment. A therapeutic gene can be delivered into a cell by viral and non-viral methods, and such gene will direct the cell to produce its own specific type of medicine or remedy (protein, RNA or metabolite). After the first human gene therapy trial in 1989 nearly 1340 clinical trials comprising of some approved, some completed and some ongoing trials for around 100 genes are reported.

In the last few decades genetic engineering have been enormously successful in identification, characterization and diagnosis of novel genetic disease. Now a days genes are successfully and safely transferred into animals and humans. An effective gene transfer approach for the treatment of many diseases is not however imminent: low transfection efficiency, presence of barriers and poor maintenance of gene expression etc. are some of the main obstacles on this therapeutic path. However gene therapy has opened new horizon in therapeutic strategy in difficult diseases including cancers.

8-3.2 Gene Therapy for Severe Combined Immunodeficiency Syndrome (SCID)

On 14 September 1990 for the first time gene therapy trial for an inherited disorder was initiated. A 4 years old patient, **Ashanthi DeSilva**, was suffering from a recessively inherited disorder, adenosine deaminase (ADA) deficiency. ADA also known as *housekeeping enzyme* has a function in purine salvage pathway of nucleic acid degradation and is expressed in various cell types . The T cells are affected due to ADA deficiency so ADA^- (ADA negative) patients suffer from severe combined immunodeficiency.

Before gene therapy was available, such patients were treated by weekly intramuscular injections of ADA enzyme conjugated to a stabilizer like polyethylene glycol (PEG) for the survival and proper function of the enzyme for some days. However it had certain limitations: viz; it does not provide full immune reconstitution and therefore it has very short shelf life

This disorder was chosen for gene therapy considering the facts below.

- The disorder is recessively inherited and the ADA expression level varies in normal population which infers that the gene expression is not tightly regulated.
- The *ADA* gene is small (32 kb, located on chromosome 20) and had previously been cloned and extensively studied.
- It was observed that allogeneic bone marrow transplantation (BMT) can cure the disorder suggesting that engraftment of T cells alone may be sufficient.
- The target cells T cells are suitable for *ex vivo* gene therapy as it is readily available and can be easily cultured.

The ADA gene therapy involves four steps:

1. Cloning of normal ADA gene into a retroviral vector.
2. Transfection of ADA recombinant into cultured ADA^- T lymphocytes derived from patient.
3. Identification of resulting ADA^+ T cells and multiplication via cell culture.
4. Re-implantion of these cells in the patient.

This approach became a model for treating various diseases by gene therapy.

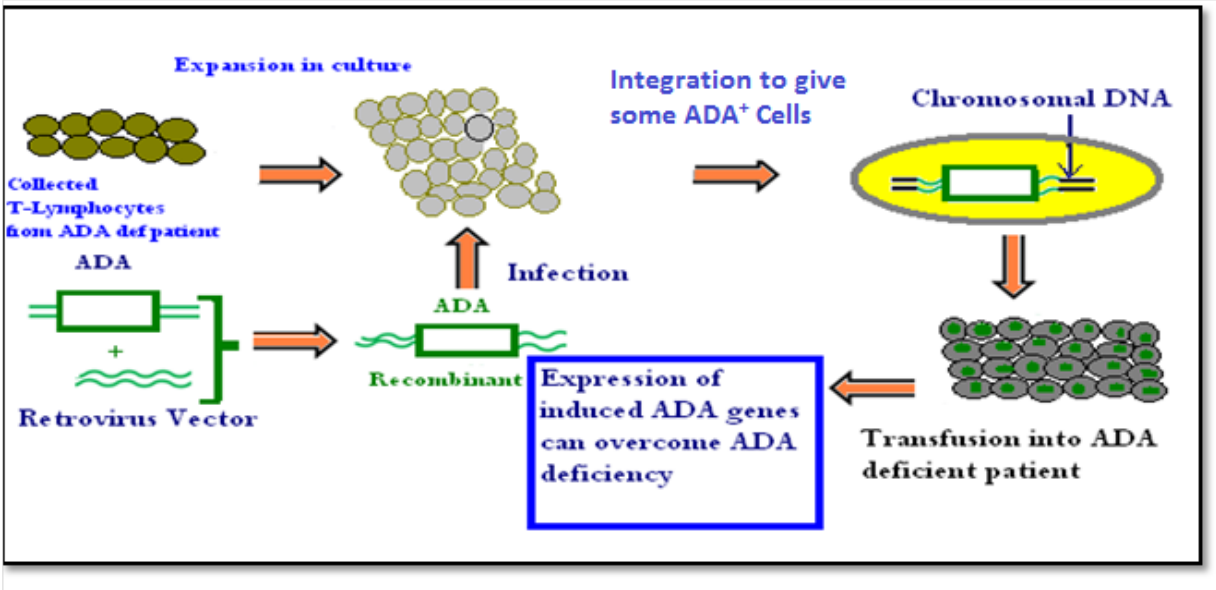


Figure 8-3.2: Ex vivo gene augmentation therapy for adenosine deaminase (ADA) deficiency.

Adapted and modified from: http://www.ncbi.nlm.nih.gov/books/NBK7569/figure/A2955/?report=objectonly.

8-3.3 CANCER GENE THERAPY

Cancer is a group of two hundred or more diseases which results due to uncontrolled growth and proliferation of normal cells. Sometimes change or mutation in the genetic material of a cell may lead to abnormal cell growth and proliferation. Cancer cells are characterized by the following six hallmarks.

- Immortality: Due to uncontrolled cell division
- Sustained Growth signals: Mutation in proto-oncogenes result in unregulated expression growth factors
- Evading growth suppressors: Due to mutation in tumor suppressor genes
- Resistant to apoptosis: Loss of proapoptotic signal due to mutation in tumor suppressor gene p53
- Angiogenesis: Formation of new blood vessels supplying nutrients and oxygen to the cancerous cells

- Metastasis: The cancerous cells can travel to other parts of the body through blood stream or lymphatic system.

8-3.3.1 Strategies for cancer treatment:

A number of strategies have been proposed for cancer treatment using gene therapy like–

- Enhancing immunological rejection of the tumor by the host.
- Decreasing tumor cell proliferation and increasing cell cycle control by restoring functions such as p53 and RB.
- Targeted poisoning of tumor cells which involves initial incorporation of an enzyme followed by administration of a pro-drug to be specifically activated in tumor cells harboring the enzyme.
- Specifically lysing tumor cells defective in the p53 or RB pathways using oncolytic viruses which are able to invade the "defective" tumor cells.

Cancer gene therapy can be divided into three broad categories: Immunotherapy, Oncolytic virotherapy and Gene transfer (Figure 8-3.3.1).

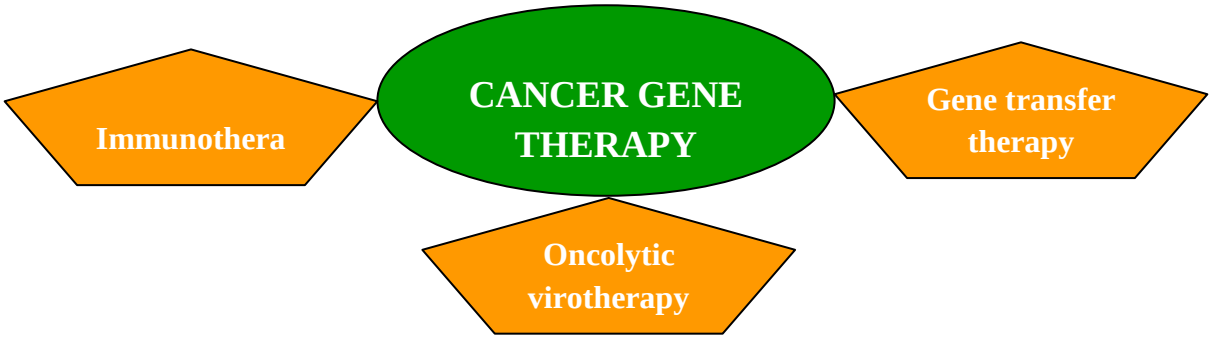


Figure 8-3.3.1. Categories of Cancer Gene Therapy

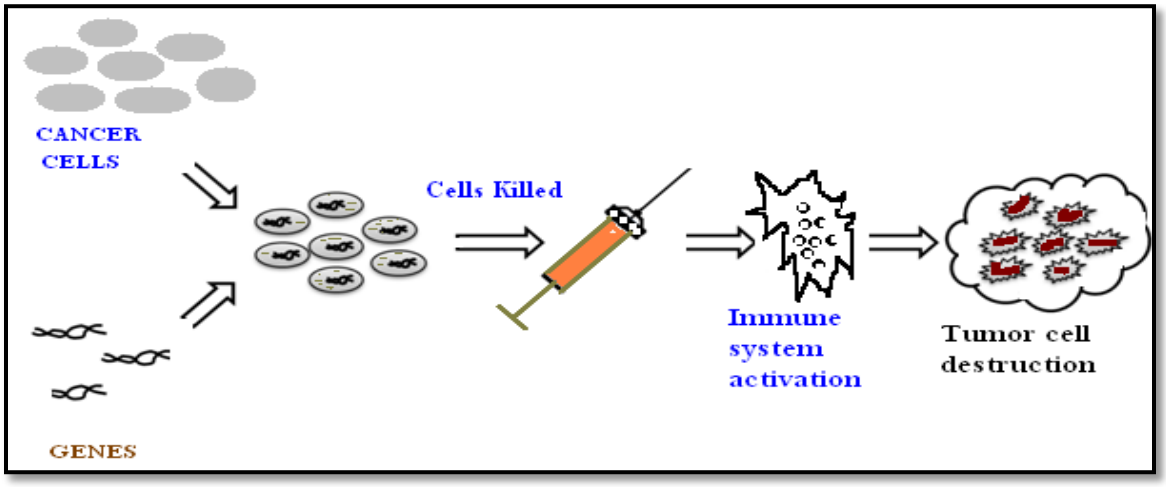
i. IMMUNOTHERAPY

Immunotherapy is based on the concept of boosting immune system to target and destroy cancer cells. Gene therapy is used to create recombinant cancer vaccines. Cancer vaccines helps in cancer cell recognition by presenting them with highly

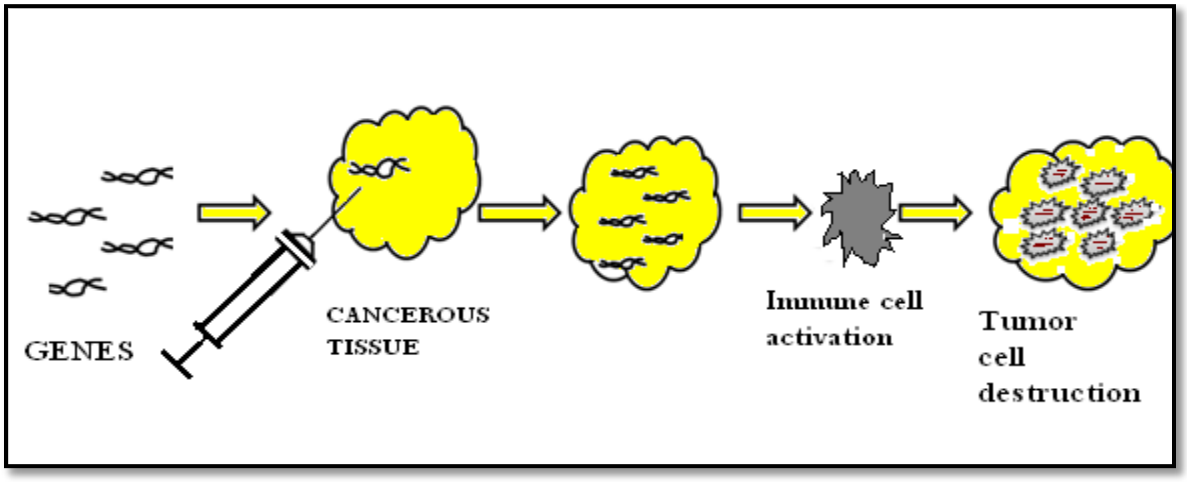
antigenic and immunostimulatory cellular components. For example, administration of reovirus to cancer cells resulted in a specific antitumor activity which could be enhanced by combination with chemotherapy and immuno- suppressive drugs.

Some immunostimulatory genes like cytokines when targeted to the tumor they produce antigens which expose the tumor cells to the immune system to be recognized and thereby promoting antitumor antibodies development.

(A) Pathway represents immunotherapy with altered cancer cells.



(B) Pathway represents immunotherapy with genes in vivo.



(C) Pathway represents immunotherapy using altered immune cells.

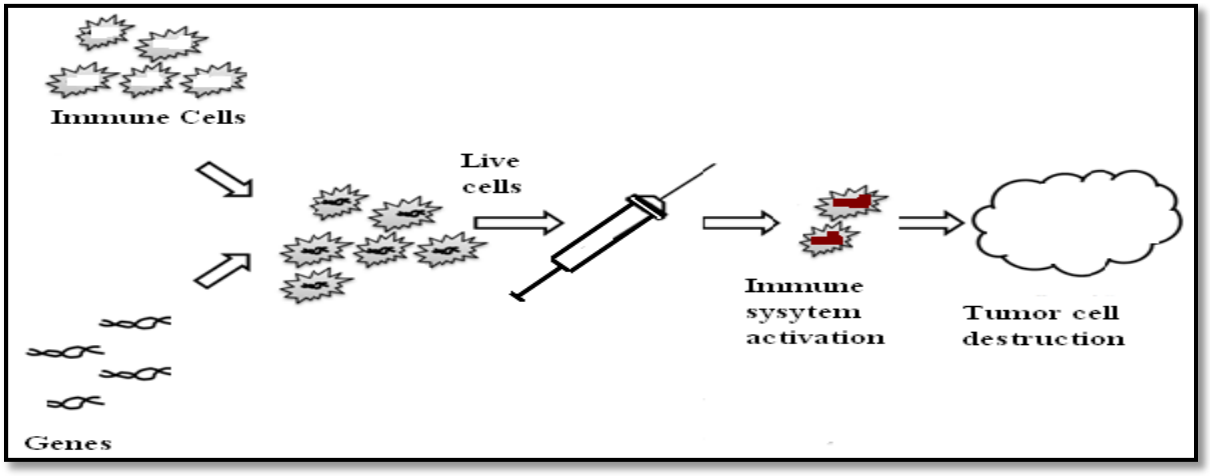


Figure 8-3.3.1(i) A, B, C: Schematic diagram of immunotherapy. Pathway A represents immunotherapy with altered cancer cells. Pathway B represents immunotherapy with genes in vivo. Pathway C represents immunotherapy using altered immune cells.

Adapted and modified from: Cross D., Burmester J.K. Gene Therapy for Cancer Treatment: Past, Present and Future. Clinical Medicine & Research 2006; 4(3): 218-227.

ii. ONCOLYTIC VIROTHERAPY:

In this technique genetically engineered viruses for example vaccinia, adenovirus, herpes simplex virus type I, reovirus are used to kill the cancer cells. The potential of this technique is popularized after the initial phase I trials for several vectors.

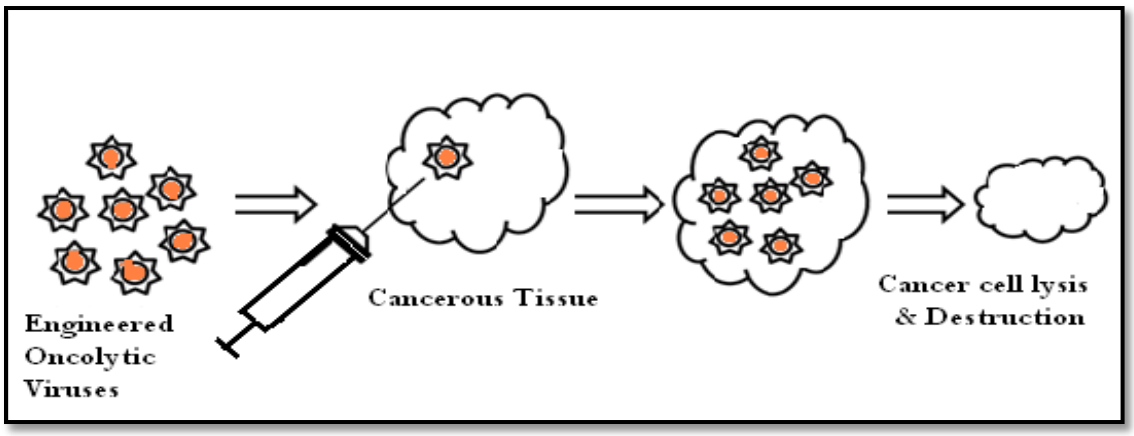


Figure 8-3.3.1(ii). Schematic diagram of oncolytic virotherapy.

Adapted and modified from: Cross D., Burmester J.K. Gene Therapy for Cancer Treatment: Past, Present and Future. Clinical Medicine & Research 2006; 4(3): 218-227.

iii. GENE TRANSFER

Gene transfer is the introduction of candidate genes using viral or non- viral vectors into a cancerous cell or the surrounding tissue to cause cell death or slows down the growth of the cancer cells while remaining innocuous to the rest of the body. Viruses used for this purpose, include vaccinia, adenovirus, herpes simplex virus type I, reovirus and Newcastle disease virus.

Example: **Gendicine** is a modified adenovirus *(produced by Shenzhen SiBiono GeneTech, China)* that delivers the p53 gene to cancer cells and has been approved for the treatment of head and neck squamous cell carcinoma in certain countries.

Non-viral methods include transfer of naked DNA and oligo-dendromer DNA coatings using electroporation as a mode of gene delivery.

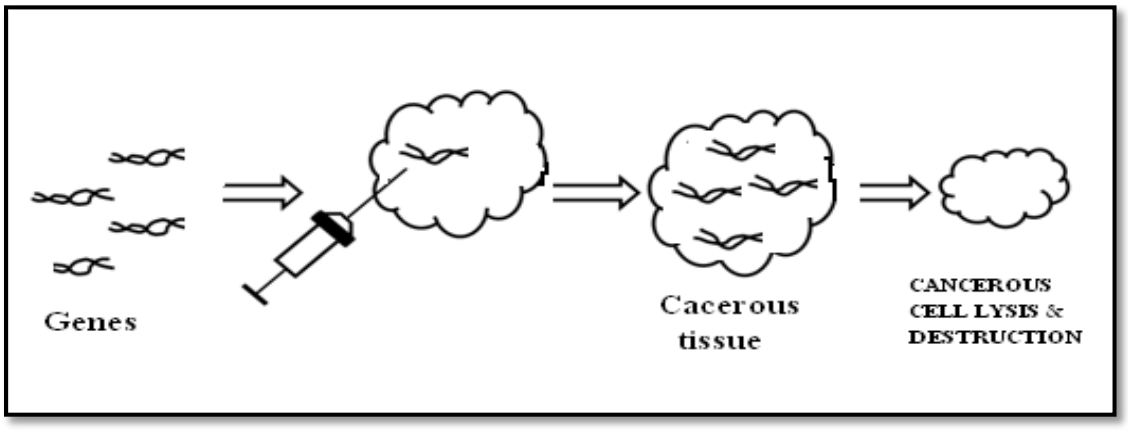


Figure 8-3.3.1E. Schematic diagram of gene transfer therapy.

Adapted and modified from: **Cross D., Burmester J.K.** ***Gene Therapy for Cancer Treatment: Past, Present and Future. Clinical Medicine & Research*** **2006;** ***4(3): 218-227.***

Cancer is a complex disease and so is its treatment which currently uses multiple methods including surgery, chemo-, radio-, immune- therapy etc. It is visualized that as the various gene therapies mature, they would mostly be used in combination with current treatments to help make cancer a manageable disease.

8-3.4 GENE THERAPY FOR MUSCULAR DYSTROPHY

Muscular dystrophies are a group of inherited disorders characterized by progressive muscle weakening often occurring in early childhood. The most common is Duchene muscular dystrophy (DMD), which affects 1 in 3500 male births. DMD is a severe X-linked recessive disorder. Generally males are affected and suffer from progressive muscular deterioration which and become wheel chair dependent early in their life. MD arise from defects in the dystrophin gene which encodes a large cytoskeletal protein called dystrophin important for membrane stability and force transduction from muscle fibers.

As most types of muscular dystrophy arise from single-gene mutations, gene therapy, involving replacement or modification of a gene, is emerging as a promising approach for treatment. However challenge remains in delivering therapeutic genes to the vast majority of muscles tissues in the body which makes up >40% of the body mass.

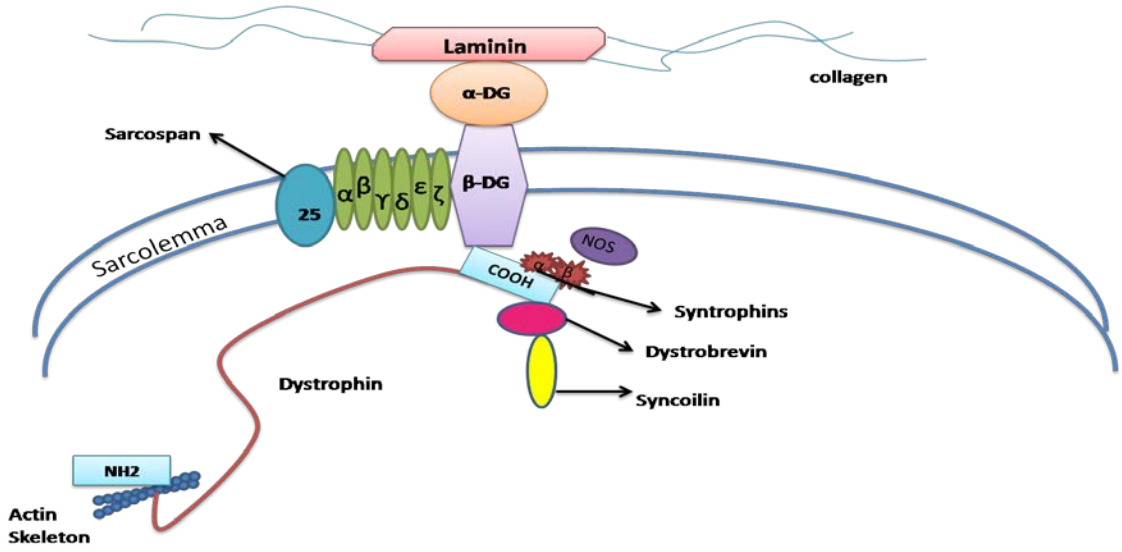


Figure 8-3.4. Dystrophin and the dystrophin–glycoprotein complex in muscle. Dystrophin is a cytoskeletal protein that links the γ-actin filaments to the extracellular matrix via the dystroglycan/sarcoglycan complexes which is a subcomplex of DGC. Defect in dystrophin results in destabilization and loss of the DGC (*dystrophin glycoprotein complex*).

Adapted and modified from: Tejvir S. Khurana & Kay E. Davies (2003) Pharmacological strategies for muscular dystrophy Nature Reviews Drug Discovery 2, 379-390

The sarcolemmal dystrophin complexes with the subsarcolemmal glycoprotein to form a large complex called dystrophin glycoprotein complex (DGC). This complex establishes a link between the cytoskeleton and plasma membrane with the N terminal binding to cytoskeleton and C terminal to the later. Any mutation in DMD genes results in a defective dystrophin which results in defective DGC leading to instability in the myofibril membrane and finally resulting in cardiomyopathy and muscular dystrophy.

In MD adenoviral vectors are chose over oncoretroviral vectors to deliver genes *in vivo* to muscle fibres as oncoretroviral vectors are inefficient to infect the post mitotic adult skeletal muscle fibres. The coding sequence of dystrophin is very large (~14kb) form which the central part seems not to be important.

Alternative strategy involves over-expression of compensating genes either through gene transfer or by up regulating expression using small molecules. Utrophin the paralog of dystrophin is highly expressed in the fetal period. Over expression of utrophin, confers a protective effect.. Encouraging result like improved muscle function are obtained in mice with dystrophin deficiency when utrophin transgenes are over expressed. Now researchers are trying to discover new molecule which can upregulate the utrophin gene expression upon administration.

8-3.5 GENE THERAPY FOR RESPIRATORY DISEASES

8-3.5.1 Emphysema

Emphysema is caused due to damage in the lungs that leads to shortness of breath. Alpha1-antitrypsin, which normally is secreted by hepatocytes and macrophagesinhibits trypsin and as well as blood protease elastase. Point mutation in the alpha1-antitrypsin gene leads to lung tissue degradation due to the proteolytic activity of elastase enzyme which eventually results in emphysema. Macrophages are the targets for gene therapy to cure emphysema. I

A team at Boston University's School of Medicine used a **lentivirus** to introduce a functional version of the antitrypsin gene into lung's alveolar macrophages of mice with alpha-1 antitrypsin deficiency and was successful in treating the condition for two years.

Liposome- mediated gene therapy is an approach to target gene into the lungs. Cationic liposomes are microscopic lipid (fat-based) vesicles which bind to DNA and facilitate cellular uptake. These are the most efficient non-viral gene delivery vector.

Methods using cationic detergents that self-assemble on the DNA backbone and form a cross-linked lipid have been found to improve DNA transfection. Toxicity of the cationic lipoplexes can be reduced by lessening the cationic part.

8-3.5.2 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder. It occurs due to mutations in *CFTR* (*Cystic fibrosis transmembrane conductance regulator)* gene, which encodes a cAMP-regulated chloride channel and results in defective transport of chloride ions and water across cell membranes of epithelial cells resulting in increase in sodium chloride (salt) concentration in bodily secretions. Higher Na^+ ion concentration outside the cells lining lungs, pancreas and other organ causes secretion of a very thick and sticky mucus that makes the organs prone to many chronic infections

Lungs are targeted for gene therapy as the defect is primarily expressed in lungs. There is no established protocol to culture lung cells in the laboratory so *in vivo* gene therapy is adopted. In some clinical trials through broncoscope or nasal cavity adenoviral vectors and liposomes carrying *CFTR* minigene are used for gene delivery.

In the first adenoviral protocol in 1993, high doses of recombinant adenovirus caused health problems which caused safety issues regarding the technique and encouraged to verify the maximum dose

The disadvantages in using adenoviral vector are

- Absence of adenoviral receptor at the apical side human epithelial cells lining of the alveolar sac results in low transduction.
- Small packaging capacity of adenoviral vectors is a limiting factor as human *CFTR* gene is large and has to be linked with strong promoters.
- To induce immune response repeated administration is needed which has to be avoided

Serotype 2 AAV vectors used in later studies could not be re-administered due to stimulation of immune reactions. In contrast non-viral vectors (polyethylenimine) were found compatible with repeated administration, but their efficiency was unpredictable and expression of transgene was generally low.

The presence of unmethylated CpG motifs on the plasmid DNA caused flu-like symptoms Liposome-based gene therapy is regarded as safer procedures, but as stated earlier the efficiency of gene transfer is much lower. CF gene therapy has been remained ineffective even though immense research is going on.

The *UK CF Gene Therapy Consortium* is working on assessment of repeated administration of non viral vector for improvement of CF lung disease . Lentivirus is suggested to be able to evade the immune system allowing for repeated administration and long lasting expression. Non-viral vector can be promising approach as it can carry the large *CFTR* genome extrachromosomally.

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MODULE 8: LECTURE 4

CHALLENGES AND FUTURE OF GENE THERAPY

8-4.1 Introduction

Gene therapy is one of the fastest developing fields of medicine as of today. About 500 clinical trials are being carried out globally in order to evaluate the efficiency of this treatment in different diseases. Gene therapy technique uses DNA to treat disease and it therapeutically alters the gene expression in a cell. Therapeutic proteins are produced by cloning the DNA into a vector and then by expressing that DNA inside the target cell of the patient which attempts to treat and address specific ailment. Gene therapy has gone through a series of triumphs and failures, which have generated both optimism and frustration at the same time.

8-4.2 Challenges in gene therapy

Although modification of germ-line cells at gene level offers the possibility of permanently eliminating certain genetic diseases, important ethical concerns, including eugenics (improvement of human race by selective breeding) and transfer of undesirable trait or side-effects to the patients' descendents, currently prohibit its development and therefore only somatic gene therapy is in progress. Gene therapy poses one of the greatest technical challenges in modern medicine especially since it is quite hard to introduce new genes into cells of the body partly due to a numbers of barriers. It faces numerous challenges as discussed below.

8-4.2.1. Gene delivery and activation

The prerequisite of gene therapy is the delivery of vectors to the target cells at the target area of tissue. The gene should be in higher concentration at the site of target cells so that the cells can easily take up the corrected or wild gene. When the gene reaches its destination it has to be activated to produce the desired protein encoded by that specific gene. However, such gene delivery and activation are the biggest obstacles faced by the gene therapy enthusiasts.

As far as choosing the best vector, there is no one "perfect vector" that can treat every disorder. Like any type of conventional medical therapy, gene therapy vector must be customized or tailor made to address the unique problem of the disease. Most commonly used gene therapy vectors are viral vectors which suffer from various disadvantages described in lecture 1of module 8.

8-4.2.2. Introducing changes into the germ line

As told already targeting a gene to the correct cells is crucial for the success of any gene therapy. It is important to ensure that the gene of interest is not accidentally incorporated into the wrong cells which would cause the gene therapy to be inefficient and could develop a new health issues for the patient.

For instance, improper targeting could lead to incorporation of the therapeutic gene into patient's germ line, or reproductive cells that produce sperm and eggs. In such circumstances, the patient would pass on the delivered gene to their next generation. This could prevent the disease in future generation or it may result in unwanted consequences in foetus development. It has also ethical concerns as it involves genetic manipulation of an individual who is not yet born.

8-4.2.3. Immune response

Gene delivery vectors must be able to escape the host's natural immune surveillance systems. Otherwise it can lead to serious illness or even death. One patient, who had a rare liver disorder, participated in a gene therapy trial at the University of Pennsylvania during 1999. But unfortunately, he died due to complications from inflammatory response after receiving a dose of experimental adenovirus mediated gene therapy for which US banned gene therapy trials for some time.

8-4.2.4. Disrupting important genes in target cells

A gene that is introduced into any group of cells ideally needs to remain intact and continue to function inside the cell. For this the freshly introduced gene must integrate into the cells existing nuclear DNA which is a random process i.e. not site-specific that may lead to integration of foreign gene within the existing nuclear genes causing disruption of those genes in the cells.

Let us see the case of gene therapy:

Gene therapy is used to treat a number of children with X-linked **Severe Combined Immune Deficiency (SCID)**. SCID patients, practically have no immune protection against microorganisms like bacteria and viruses. To escape infections and illnesses, they must live in a completely germ-free environment that is often impractical. A clinical trial for a gene therapy to restore the much needed function of a crucial gene, γ- c, to cells of the immune system was introduced. γ- c gene encodes for a subunit of cytokine receptors (interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15). Defect in this gene leads to with X-linked Severe Combined Immune Deficiency (SCID) due to blockage of T cell differentiation**.** This treatment appeared to be quite successful in restoring immune function in the recipient children with SCID.

But later, two of these children developed leukemia/blood cancer. It was found that the leukemia occurred because the newly transferred γ-c gene had incorporated into the wrong location, interrupting the function of another important gene that normally helps regulate the rate of cell division. As a result, the cells started dividing in an uncontrolled

manner, causing the leukemia. Although doctors could treat the children successfully with chemotherapy, the development of leukemia attributable to gene therapy raised safety-related issues. The pioneering clinical trials for SCID-X1 gene therapy provided convincing proof of efficacy of gene therapy, however the unexpected cases of leukemia has underlined the risk of *insertional mutagenesis* and adverse effect that must be addressed.

8-4.3 Future of Gene therapy

Gene therapy has not yet been realized to its full potential in clinical applications. The technology is still in developmental stage and yet to be developed. Between 1990 to 2000, several thousand patients were treated by gene therapy, mostly without long-term success.

The future successes of gene therapy also depend on the advancements in other relevant fields, such as medical devices, cell therapies, protein therapies and nanotechnology.

8-4.3.1. Medical devices

Medical advancements through innovative Medical Devices combined with biological proteins or drugs have become an exciting new area in treating patients with chronic diseases.

Advances:

- Stents containing proteins or drugs have been in use to treat cardiovascular diseases;
- Growth factors containing Collagen scaffold have been used to stimulate bone growth;
- Sustained release of *Nerve Growth Factor* attached to biodegradable *polymer microspheres* for central nervous system cell stimulation and growth
- Bone and cartilage cells have been stimulated to grow with growth factors in representative matrix samples.

- Development of gene releasing scaffolds is also being attempted

In future, advances in these fields are likely to facilitate gene therapy. On the other hand, advances in the field of gene therapy will improve the treatment outcome in patients and may have stimulatory effects on growth of medical devices.

8-4.3.2. Protein therapies:

Protein therapies for diseases have been evolving due to better understanding of the molecular basis of many diseases. **Insulin** for diabetes & human **erythropoietin** for treating anaemias produced in cell culture using recombinant DNA technology (commercially available as Epogen) and various **cytokines** available for stimulating stem cells are few of the notable examples. The manufacture of such therapeutic proteins has been the *rate-limiting step* in these new classes of therapies into the clinic.

In future, better understanding of the molecular basis of human diseases will give additional therapeutic proteinaceous products for ready to use clinical applications.

8-4.3.3. Cell therapies:

The role of *stem cell therapy* offers a huge potential to treat many human diseases. However to meet the various challenges, the following are essential;

- Fundamental understanding of the intricate biology of the stem cell growth and differentiation.
- Understanding the regulatory cascade of the signal transduction pathways. This would enable the switching on and off the subset of regulatory genes for ensuring selective stem cell growth and differentiation.

8-4.3.4. Regenerative medicine

- According to ESF, Regenerative medicine is method to develop a functional tissue which can repair the tissue or organ which has lost its function due to any damage or disease or age or congenital disorder.
- A corresponding increase in the incidence of non-communicable diseases like cardiovascular diseases and cancers, due in part to an increasing older population in industrial societies that are vulnerable to age related DNA damage and cell degeneration.
- Since, now it is possible to grow some organs in the laboratory through tissue culture approach, regenerative medicine has the potential to solve the problem of many patients who require life-saving organ or tissue transplantations.
- Deriving benefits of the biotechnological and medical advances of the last century, we can only expect many novel treatment options for chronic human diseases with innovations like cell therapies, new molecular entities (protein therapies and gene therapies) and small molecules e.g. nano-molecule mediated therapy.
- Gene therapy based regenerative medicine involves direct delivery of the therapeutic gene to the patient or genetic modification of embryonic stem cells or differentiated cells or adult stem cells and later reintroduced into the patient body. One of the successes of this application is the gene therapy of hematopoietic cells of the patient having congenital immunodeficiency disorder.

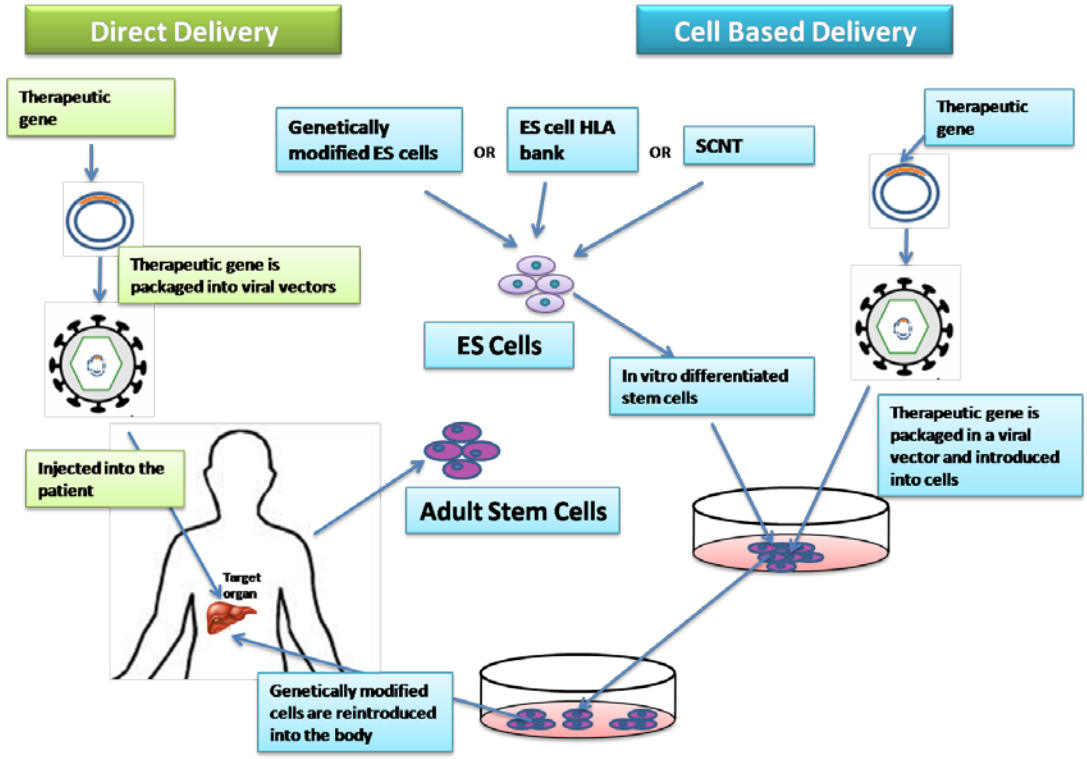

Figure 8-4.3.4: Applications of gene therapy in regenerative medicine by direct delivery and cell based delivery

Adapted and modified from*: *http://stemgenex.com/blog/stemcellgenetherapytorevolutionizemedicine.html

8-4.4 Meeting the challenges in gene therapy

Some issues that need to be addressed before gene therapy could be successful are:

1. Identifying the correct therapeutic gene to inhibit disease progression.
2. Identification of key target genes critical for the disease pathology and progression. Inhibition of a single target gene sometimes is not sufficient to inhibit the disease as there may be alternate pathways compensating the function.
3. Optimal trans-gene expression for suppressing the offending/target gene.

   For optimized trans-gene expression two important components are required: promoters and enhancers to determine the time duration of trans- gene expression in the target cell or tissue. There are two types of promoters: constitutive or inducible.

The *constitutive promoters* can be either of viral origin (e.g. Cytomegalovirus) or tissue specific promoters, such as melanin for melanoma or the prostate specific antigen (PSA) for prostate cancer. *Inducible promoters* can be induced by inducers (small molecules or hormones) and leading to transient expression of the transgene.

Enhancer sequences are inserted to *upstream* portions of the promoters to increase the *trans*- gene expression ability by 2-100 times. This is required in the case when the gene product needed in very high concentrations inside the target cell. The trans-gene expression duration will depend on the cell's requirements and nature of the product.

4. **Delivery of therapeutic product** to the target tissue at an efficacious dose:

This depends upon the disease. In cancer cells, the duration of expression may need to last for only a short time up to 30 days. In contrast, genetic diseases may require long-term expression from months to years that may require highly effective transfection and integration of the gene.

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