Module 1 Introduction

Lecture1

Introduction (Part I)

Hershey and Chase experiment in 1952 proved that the DNA is a genetic material; the finding revolutionized the field of biology. In 1953, the DNA double helical structure was elucidated by Watson and Crick based on the X-ray diffraction image by Rosalind Franklin. Subsequently the genetic code was elucidated by Nirenberg and Khorana.Further Maniatis and coworkers cloned the globin gene. All thesediscoveries opened a large arena for manipulating the animal genome and ask the question whether we can treat any heritable disease by using molecular biology tool. The field of gene therapy emerged as a new area for the researchers around the world.

A hereditary unit that consists of sequence of DNA which occupies a specific location on a chromosome and determines a particular characteristic of a living organism is called as **Gene**. Fluorescence in situ hybridization (FISH) is a method by which a gene is localized with the help of a fluorescent dye in a chromosome. The gene can be visualized for its correct position and location in the chromosome. Any deviation from the normal can be suspected for a potential genetic disease.

All the living cells in the universe are divided into either a prokaryotic (primitive nucleus) or a eukaryotic cell (true nucleus). Although they are different from each other in many aspects but both contain genes that code for specific polypeptides required for their perpetuation in the environment.

Table 1.1 Comparison of a eukaryotic cells with a prokaryotic cell:

Cell organelle	Eukaryotic cell	Prokaryotic cell
Nucleus	Present	Absent
Cell type	Usually multicellular	Unicellular
True membrane bound nucleus	Present	Absent
Lysosomes and peroxisomes	Present	Absent
Endoplasmic reticulum and mitochondria	Present	Absent
Ribosomes	Larger	Smaller
Cell wall	Only in plant cells and fungi Chemically simpler	Chemically complex
Plasma membrane with steroids	Yes	Usually no

Gene therapy is a process that involves introductionof genetic material into a person's cell to cure or prevent the disease condition. Researchers around the world are studying gene therapy for a number of diseases, such as cystic fibrosis, hemophilia, cancer, and even influenza, through a number of different approaches.

Some facts:

Human genome project has completed in the year 2003. Human genome contains about 3 billion nucleotides. The entire genome containsmore than 35,000 genes.

Basic process of gene therapy involves one of the followings

- Introducing a new specific gene into the body to combat a particular disease.
- Interchanging a disease causing gene with a healthy copy of same gene.
- Inactivating, or "knocking out," of an impaired gene that is not carrying normal function using engineered nucleases such as zinc finger nucleases, engineered I-*Crel* homing endonucleases or the nucleases obtained from TAL effectors.One of the best examples of gene-knockout mediated gene therapy is the knockout of the human CCR5 gene in T-cells to control HIV infection.

The method of gene transfer and its expression is termed as transduction. A gene can be transferred to an individual using a carrier known as a "vector". Despite the fact that the principle of gene therapy is quite simple but the outcome depends mainly on the vehicles or vectors through which the gene transfer is carried out. Practically gene transfer vectors can be divided either into synthetic or virus based gene transfer systems. With the passage of time many gene transfer vectors have been introduced. Viruses such as adenovirus, adeno-associated virus, and lentiviruses are the most common types of vectors used in gene therapy. The viruses are altered in order to make it safe before using them as a vector for gene therapy. However some risks still exist with gene therapy. The method is still in its inception, but it has been used with varying degree of success.

Facts:

The first gene therapy experiment was conducted on a 4-years-old girl at National Institute of Health, USA in 1990. She was suffering from a genetic disease having deficiency of adenosine deaminase enzyme required for the proper metabolism of purine. Any malfunctioning of adenosine deaminase leads to severe combined immunodeficiency in affected patients. The white blood cells of the girl was taken out and corrected with a human gene using retrovirus as a vector.

In general, an altered gene can be inserted into a patient using a viral or a non-viral vector. Essentially all cells of the body contain genes, making them suitable for gene therapy. Broadly cells can be divided into somatic (most cells of the body) or germline (eggs or sperm). It is possible to manipulate either somatic cells or germ cells in terms of genetic composition and it also serves as the basis of gene therapy. Gene therapy using germ cells result in permanent changes that are subsequently passed on to next generation. Gene therapy using somatic cells are not passed on to the next generation. Somatic cell gene therapy is safer than the germ line gene therapy because it targets only the affected cells in the patient's body. Somatic cell gene therapy can be done exterior (where cells are modulated *in vitro* and then transplanted back) or interior (where genes are changed *in vivo*).

Gene Doping:

The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to improve athletic performance is defined as Gene Doping by the World Anti-Doping Agency (WADA).

1.1 Qualities of a suitable vector

1) Anoptimal vector should be one that can be made available in a highly intense form using an ideal and reproducible production plan.
2) The vector must be competent of directingthe cell type most relevant for the disease, either it be multiplying or non-multiplyingcells.
3) Those vectors that can accomplish site-specific integration are more preferred and desirable in case of insertional mutagenesis.
4) A perfect vector should be harmless and should not cause any noxious effect on human health.

Synthetic gene delivery systems rely on direct delivery of genetic message into a specific cell and involve injection of exposed DNAand encapsulation of DNA with liposomes (cationic lipids mixed with nucleicacids), and nanoparticles. Even though this method displays low toxicity and is formed in abundance, gene transfer in common is weak and often temporary.The synthetic gene delivery transfer vectors are

also known as non-viral vectors. It is possible that the time to come may witness advanced gene therapy protocols for synthetic gene delivery systems based on the experiences from non viral vector systems.

Viral vector systems are established on replicating viruses with either RNA or DNA genomes that have the potential to deliver genetic message into the host cell. These vectors are either of integrating or of non-integrating type. Integrating viral vectors exhibit constant expression of the defective gene product. Overall, genomes of replicatingviruses consist of coding regions and *cis*-acting regulatory elements. Thecoding sequences include the genetic message of the viral, structural and regulatoryproteins and are needed for division of infectious viruses, whereas *cis*-acting sequences are important forwrapping of viral genomes and unificationinto the host cell. To generate a replication-defective viral vector, the codingdomains of the virus are substituted by the genetic message of a therapeuticgene, leaving the *cis*-acting sequences intact.

Monogenic disorders	Cancers
Cystic fibrosis	Gastrointestinal tumors
Hemophilia	Nervous system tumors
Severe combine immuno deficiency	Sarcoma
Familial hypercholesterolemia	Respiratory system tumors

Gene therapy offers promising approaches towards treating many incurable diseases. Many hurdles and challenges are there for a sustained gene therapy approach and scientists around the globe are working to increase the efficacy of gene delivery vectors.

Setback history:

Jesse Gelsinger an 18 year boy who was suffering from a genetic disorder of liver named ornithine transcarbamylase deficiency died on September 17th, 1999 during a clinical trial of gene therapy. Gelsinger was treated at the University of Pennsylvania using adenovirus vector containing a corrected copy of the gene. His death was a great set back to the field of gene therapy.

Lecture 2

Introduction (Part II)

2.1 Non-viral vectors in gene therapy:

Non viral vectors are biocompatible moieties which can be produced in large scale, easy to manipulate and are easy for cell or tissue targeting. The only disadvantage of non-viral vector is its reduced transfection efficiency in cells or tissues. Non-viral delivery systems utilize different genetic materials such as antisense oligonucleotide (*AON*), plasmid DNA, small interfering RNA (*siRNA*), short hairpin RNA (*shRNA*), and micro RNA (miRNA) that works on electrostatic interaction. On the other hand cationic vectors such as lipid and polymers help in the formation of lipoplexes and polyplexes.

2.2 Liposomes in gene therapy:

The name liposome has been derived from two greek words "lipos" meaning fat and "soma" meaning body. A liposome is a tiny bubble (vehicle) made out of the same material as a cell membrane. Liposomes can be filled with DNA and can be used to deliver for cancer and other diseases. Membranes are usually made of phospholipids which are molecules that have a head group and a tail group. Head is hydrophillic and tail is long hydrocarbon chain and is hydrophobic. Usually phospholipids are bilayer stable membranes. During packaging of the polynucleotide material in liposome, a number of structures are formed. Each structure formed is the most energetically favorable conformation based upon characteristics of specific lipids used. A dependent term known as the structure-packing parameter can be used to suggest what shape the amphiphile will take depending on the size variables.

2.3 Lipoplexes and polyplexes in gene therapy:

To improve the delivery of DNA into the cell, it must be protected from damage and its entry into the cell must be facilitated. For this lipoplexes and polyplexes are used because of their ability to protect DNA from undesirable degradation during the transfection process. Plasmid DNAs are covered by lipids in an organized structure like a micelle or liposome. When the organized structure is

complexed with DNA, it is called lipoplex. There are three types of lipids: cationic, anionic and neutral. Cationic lipids are most preferred for gene delivery because these are positively charged and thus complex well with negatively charged DNA and interacts with the cell membrane, endocytosed by the cell and finally release the DNA.

2.4 Naked DNA in gene therapy:

The simplest non-viral gene delivery system uses naked DNA as a vector. Direct injection of free DNA into certain tissues specially muscles produce high levels of gene expression.It is particularly applied to cancer tissues where the DNA can be injected either directly into the tumor or can be injected into muscle in order to express tumor antigens that might function as a cancer vaccine. It can also be used to treat genetic diseases in the tissues that are available for direct injection such as skin. But the expression is usually non uniform and fails to correct the underlying histological and functional abnormalities of the disease. Though it leads to gene expression but level of expression is much lower than with either viral or liposomal vectors. It is unsuitable for systemic administration due to the presence of serum nuclease. Therefore its application is limited to muscle cells and skin.

2.5 Immunotherapy:

Immunotherapy comprises of majority of the gene therapy trials for cancer. It has been well established that cancer cells are weakly immunogenic because they are self and by virtue of many ways to down regulate host immune response. Most of the studies related to cancer gene therapy involve manipulation of tumor cells *ex vivo* to enhance the production of interleukins, interferon gamma and tumor necrosis factor. Moreover irradiated tumor cells can be used as a vaccine in many types of cancers. Currently, antigen presenting cells such as dendritic cells isolated from a patient are modulated to enhance the immune response against cancer.

2.6 Gene therapy for infectious diseases:

Gene therapy may be an attractive tool for the treatment of many important infectious diseases such as HIV. The disease is affecting more than 15 million people worldwide causing decrease in CD4+ cells in the body making patient immunocompromised. The current approaches include targeting siRNA to inhibit virus replication, suicidal gene therapy using adenoviral vectors, and transducing HIV coat protein into patients for inducing immune response. In addition, cloning the immunogenic gene into a suitable plasmid expression vector under the control of tissue specific promoter can be used as a DNA vaccine to elicit immune response against many viral and bacterial diseases.

2.7 Virosome:

Virosome is a drug or vaccine delivery system. It is a virus-like particle that acts as a vaccine carrier and adjuvant thereby acting as an immune enhancing system. Vaccines that are manufactured using virosome technology show high efficacy with high purity and hence it is a safe and an effective way to vaccinate infants and adults. Virosomes are reconstituted viral envelopes including membrane lipids and viral spike glycoproteins but devoid of viral genome. They are highly effective as vaccine antigens and adjuvants because they stimulate humoral immune response because of the presence of viral glycoproteins. The main advantage of virosomes over other drug delivery system like liposomal and proteoliposomal carrier system is that the virosomes protect pharmaceutically active substances from proteolytic degradation and low pH within endosomes. This helps the contents to be in intact form till it reaches cytoplasm. Influenza virus is most commonly used for virosome production. So, in this case it will have influenza virus' hemagglutinin (HA) and neuraminidase (NA) glycoproteins within the phospholipid bilayer membrane. Various ligands such as cytokines, peptides, monoclonal antibodies (MAbs) can be incorporated into virosome and displayed on the virosomal surface. Due to the presence of influenza HA protein virosomes have unique property of fusion activity. Virosomes bind to the cell surface and enter the cell by receptor mediated endocytosis. Due to the presence of acidic environment in the cell the contents of the virosome are released from the endosome into the cytoplasm of the target cell. Hence virosome technology is a significant technique for the delivery of virus antigens or DNA/RNA encoding

specific immune stimulatory molecules. It is target specific and can stimulate both cellular and antibody immune response to maximize protection against the targeted disease. In addition to this, they are completely biodegradable.

Lecture 3

Somatic and Germ line Gene Therapy (Part I)

3.1 Somatic cell gene therapy:

Somatic cells are all the cells present in the body except the germ cells (oocytes and spermatocytes). Somatic gene therapy is not heritable because the new DNA is inserted into a somatic cell of the body like liver cell and thus the DNA does not get into the germ cells. This transfer corrects the mutation or disorder in an individual suffering from any genetic ailment.This method used for the gene transfer can be either virus mediated or liposome mediated. Sometimes the DNA inserted gets integrated into the chromosome in the nucleus. The cells should be easy to isolate and re-implant like bone marrow cells because it continues to divide for the entire life of the individual producing blood cells. So the diseases having role in blood can be cured this way. Similarly the gene can be inserted wherever its target organ or tissue is located eg- lungs, liver, muscle.

There are certain problems with somatic gene therapy such as risk that the viral vector will combine with the cellular genome and thus infect the cell. This is called as viral escape. Another risk is activation of a proto-oncogene or disruption of an important gene. There are certain hurdles to overcome in somatic cell gene therapy. One is to target the desired cell or tissue to introduce the gene. Virus can be risky at times. Another one is to locate the right cell for example for cystic fibrosis;target should be the lungs and the gut. Aerosol sprays can be used for lungs to deliver the gene and for the gut, the gene to be delivered should be packaged such that it would swallow and still protect them from digestive enzymes until they function.

Somatic cell gene therapies comprise the implantation of cells as an *in vivo* source of any essential biomolecules such as an enzyme, cytokine or coagulation factor, transduction of activated lymphocytes, natural killer cells, and cell populations such as hepatocytes or myocytes which are involved in performing complex biological functions. Initial approach of the gene therapy was to implant the altered cell population in the patient which later on is modified by injecting retroviral vector expressing corrected gene.   The complexicities of the therapy includes interaction

of cells with other body constituents making the whole science to be tested to utmost care before using it for preclinical trial.

Figure 3.1 Schematic representation of somatic cell gene therapy:
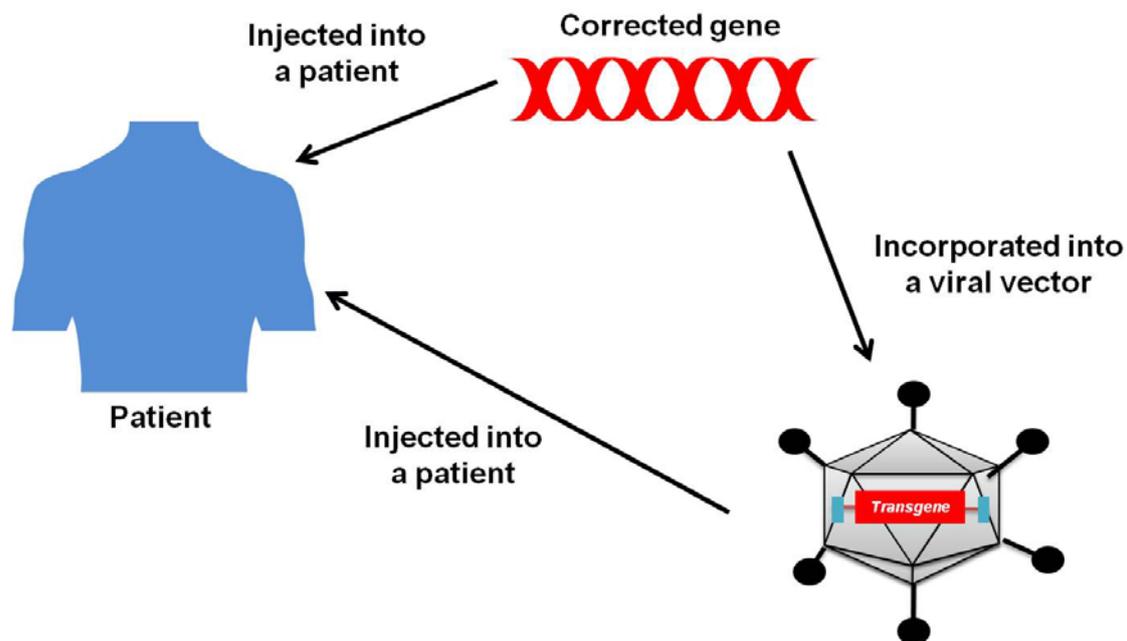
3.2 Target site for gene therapy

There are different target sites for the gene therapy

Endothelium: The advantage of using endothelium as a target site for gene therapy includes formation of new blood vessels and ease to get the gene of interest directly into the blood stream. Eg- Hemophilia

Muscle: It is easily accessible to blood stream and muscle specific promoter is well studied. Eg- Duchenne Muscular Dystrophy.

Liver: It has a capacity to regenerate and involve in a variety of functions. Thyroid binding promoter is well studied for liver specific gene transfer. Eg- Familial hypercholesterolemia.

Skin: Grafting of skin is possible and small piece can be grown to a large.

Brain: Important for nervous system related illnesses.

Lung: Airway epithelium is easily accessible organ. Eg- gene therapy for cystic fibrosis.

3.3 Transcriptional targeting approach

The specific cells or tissue can be transduced by a vector designed by using specific promoter that directs the expression of gene of interest. The gene expression of these vectors is only possible when the transcription factor proteins can bind to these promoters. Some of the promoter used for expressing the transgene is prostate specific antigen, cytomegalovirus promoter, β-actin promoter, and hypoxia inducible factors.

3.3 Transcriptional targeting approach

Lecture 4

Somatic and Germ line Gene Therapy (Part II)
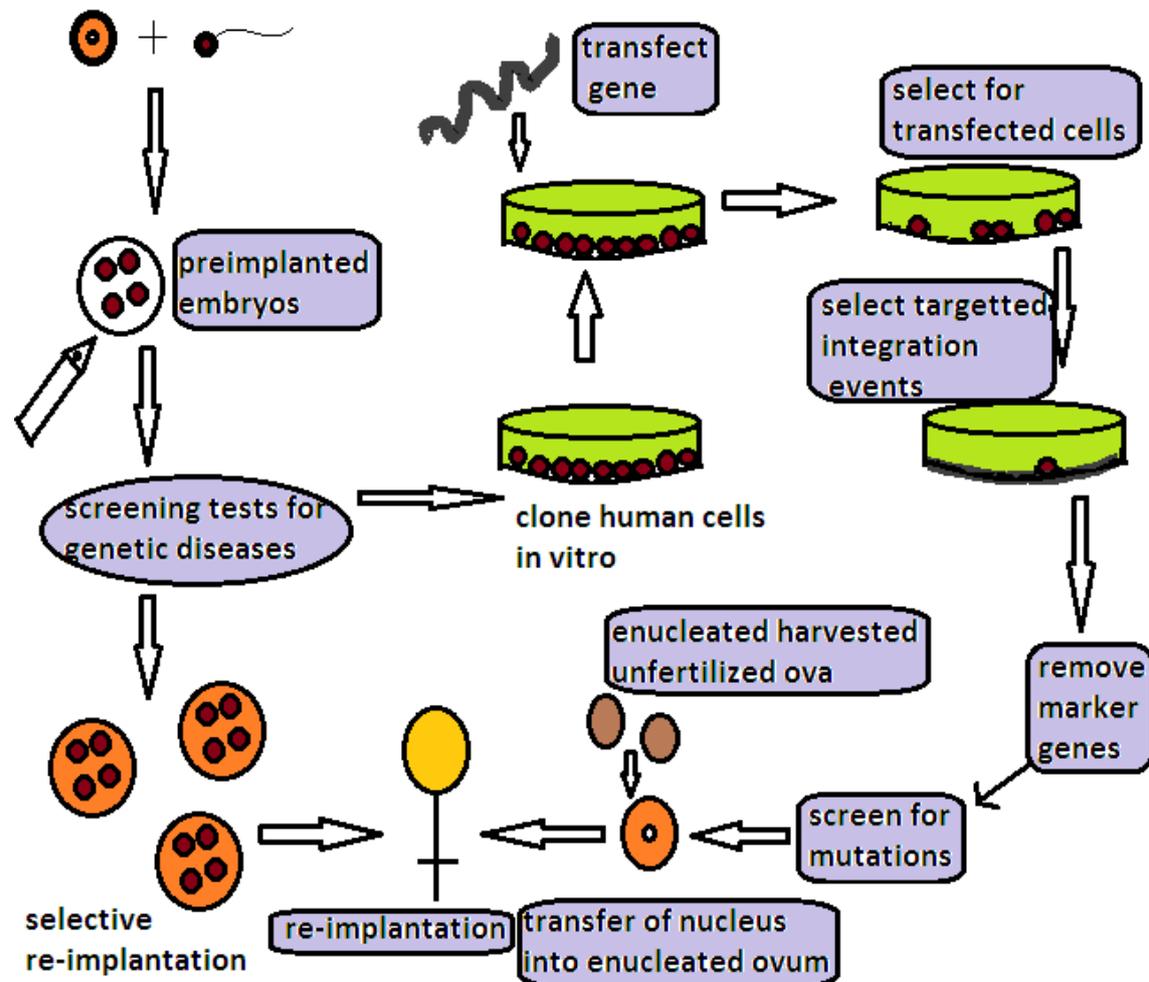
4.1 Germline gene therapy

The germ cells (sperm and egg) are manipulated in germ line gene therapy. The foreign DNA is added to the germ cells or early embryo. The advantage is that they are available outside the body easily and can thus be retrieved and modified and the inserted gene would be present in all the cells of the patient and would also be passed on to further generations. Thus it will be heritable. It is good for the people with inheritable genetic disorders. But the problem is targeting the gene to the appropriate location as it may get inserted to some other location in the genome of the cell thus causing mutation or disrupting a functional normal gene.

In germline gene therapy desirable changes or modification in the targeted genes may be done in two ways. In the first type modification of the genetic constitution of germ cells is done prior to the fertilization and hence the modifications in the genetic constitution pass on to the later generations in the form of mutation. However in present scenario this therapy is considered undesirable because of the huge risk associated with it.

In the second type of germline gene therapy changes in the genetic constitution are brought in the initial stages of blastomere.In this type the altered cells are allowed to divide and grow into an individual and only few of these cells acquire the genetic changes and remaining do not. The mutation will be inherited only if the cells acquiring the genetic changes turn into germ cells of the organism.

Germ line gene therapy has just started although experiments have been carried out in animals and some mammals. This therapy is still unacceptable ethically as no trials have been conducted on humans till date.

Figure 4.1 Schematic representation of germline gene therapy:

4.2 Steps of germline gene therapy:

1. Isolation of totipotent embryonic cells at an undifferentiated stage
2. The determination of the genetic state of the embryo
3. Embryonic stem cell expansion in culture
4. Transfer of the genetic material into embryonic cell
5. Selection of stably transfected cell
6. Targeted gene replacement
7. Removal of marker
8. Confirmation of genomic integrity
9. Transfer of the nucleus
10. Re-implantation in the mother

Figure 4.2 different delivery methods:
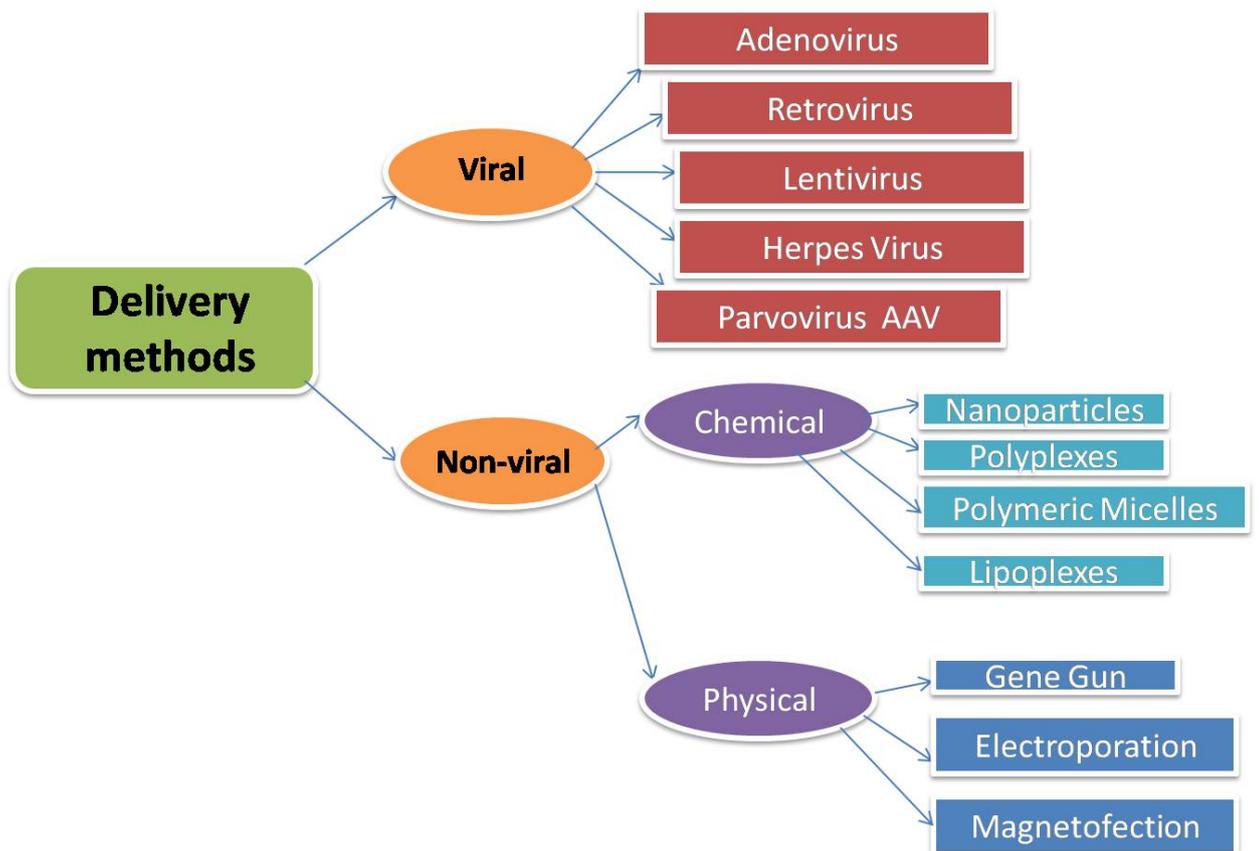
Lecture 5

Gene Replacement and gene addition (Part I)
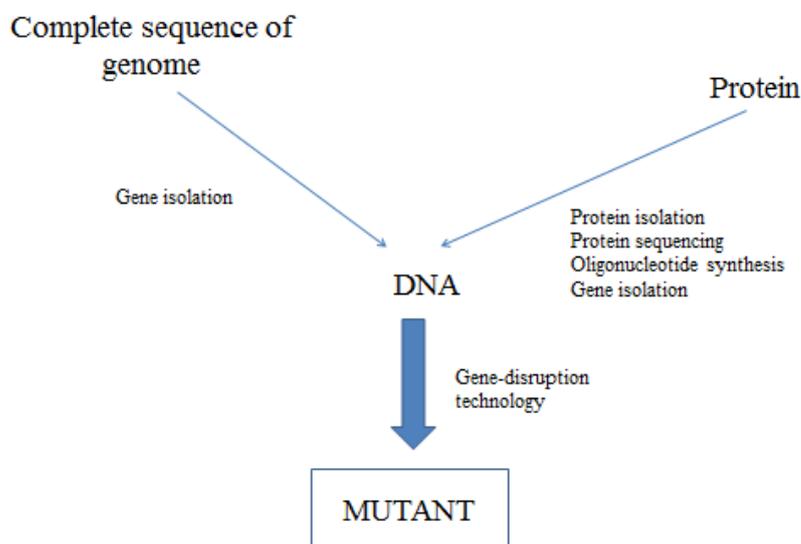
5.1 Gene Replacement:

The techniques of genome mapping, mutation, protein expression have played a great role in understanding modern molecular cell biology. However, large number of genes have been recognized and studied on the basis of the biochemical properties of their encoded protein, the sequence similarity of the encoded protein with proteins of established role, or their policy of expression during their progression. It would be very difficult to understand the *invivo* role of such genes if their mutant forms are not available. **Gene replacement** is a technique wherein a particular gene is mutated *invitro* and its native copy is then substituted by its mutant form to know its*invivo* function. Gene replacement is also sometimes known as **gene-targeted knockout**, or plainly "**knockout"**. In other words gene replacement is a technique that involves homologous recombination to replace or substitute an endogenous gene. It may include point mutations, removal of a gene/exons and even introduction of a new gene. Gene replacement or knockdown can be constant or provisional. Provisional or limited knockdown can be in terms of its restriction to a particular period during the growth of an organism or restriction to a particular tissue.

Gene replacement technique is an established method for some typical organisms and the method may vary among the wide range of species used. Overall, a construct made out of DNA is produced in bacteria. It generally comprises of targeted gene, a reporter gene and a selectable marker that helps in suitable selection. *Drosophila melanogaster* is the species of choice for many genetic experiments and in likewise species gene therapy has shown its prompt role. In some cases especially knockout experiments exhibit high frequency by using engineered endonucleases such as Zinc finger nucleases (ZFN) and homing endonucleases. In knockout mice experiments the above mentioned construct is inserted into the mouse embryonic stem cells in culture. The cells with proper insertion are then picked in order to use them for contributing to a mouse's tissue through embryo injection. Lastly, only the **chimeric**mice in which the reproductive organs are made up of modified cells are taken and selected for breeding. This step ensures that the complete body of the

mouse is based on the originally picked embryonic stem cell. In present scenario, gene knockout has now become possible in many other organisms including yeast, cattle, sheep, swine, and many fungi.

With the ease in learning the skills of genome sequencing of various model organisms mutating a native gene would probably become quite straightforward. In any case(native protein or sequenced genome) the process can be compiled in figure 5.1.

Figure 5.1 Complete genome of an organism and its application:

The other technique which involves integration of exogenous gene called a transgene into the host organism to display a new function in that particular organism is called as transgenesis.The assembly of both gene replacement and transgenesis makes use of methods for modifying cloned genes *in vitro* and then relocate them into eukaryotic cells.

One of the successful applications of gene replacement technology is the experimentation carried out in genetic engineering to eliminate unacceptable metabolic pathways in *Streptomyces clavuligerus*which is an industrial strain used for the synthesis of an antibiotic significant for the human medicine.

Important Terminology:

Chimera – It is also read as chimaera and may be defined as an organism that comprises of two or more than two varied populations of genetically distinct cells that originate from various zygotes participating in sexual reproduction. If the varying cells arise from the same zygote, the organism is termed a **mosaic**.

Lecture 6

# Gene Replacement and gene addition (Part II)

6.1 Gene addition:

In present scenario gene addition is one of the most practical approaches to gene therapy. It involves the insertion of an active copy of a defective innate gene. In the year 1990, viral vector based gene addition was successfully done to treat a patient named Thompson in the U.S. In the viral vector based gene addition Retroviruses were used at first because they were supposed and found to be most fit among all viruses for their adaptive nature for delivering genes into cells. This therapy is a promising one because it has been proven to be a substitute for gene replacement and is having a very high efficiency for curing any disease. Even though gene addition therapy is quite satisfactory but it has some benefits and drawbacks over the gene replacement therapy. Its advantages are that the cells that do not ordinarily express a specific gene can be manipulated to express that gene. For example, blood clotting factors could be synthesized in any somatic cell, making somatic tissues other than the liver convenient objectives for clotting factor gene transfer. Alternately, completely new genes might be used to cure the disease. For example, the transmission of human immunodeficiency virus (HIV) could be restricted by transfer of genes that encode ribozymes competent of degrading HIV RNA. A disadvantage of gene addition therapy is the disordered insertion of genes into the genome. Thus, the inserted genes may be erroneously expressed or may cause inappropriate expression of genes immediate to the insertion site.

The outcome of gene addition relies on the effective insertion of therapeutic genes at the suitable target spot within the host cell, avoiding any cell damage, mutations or any kind of adverse immune response. Zinc finger nucleases (ZFNs) have become essential reagents for engineering the genomes of many plants and animals including *drosophila melanogaster*. Specially constructed laboratory, ZFNs suggests a common method to deliver a site-specific double strand breaks (DSB) to the genome, and stimulate a local homologous recombination by considerable amount of magnitude. The ZFN-encoding plasmid-based approach has the efficacy to avoid all the problems linked with the viral delivery of therapeutic

genes. ZFNs drive exceedingly effective genome editing by generating a site-specific DNA DSB at a fixed site in the genome. Consequent restoration of this DSB break through the homology-directed repair (HDR) leads to targeted gene addition.

In some wet lab experiments addition of a break at a specific target site causes gene addition using a homologous donor template on using CCR5-specific ZFNs as a model system. Efficacy for a single stranded break to direct repair pathway choice may prove beneficial for some therapeutic applications such as the targeted correction of human disease-causing mutations.

Figure 6.1 Diagrammatic illustration of targeted gene correction strategy:
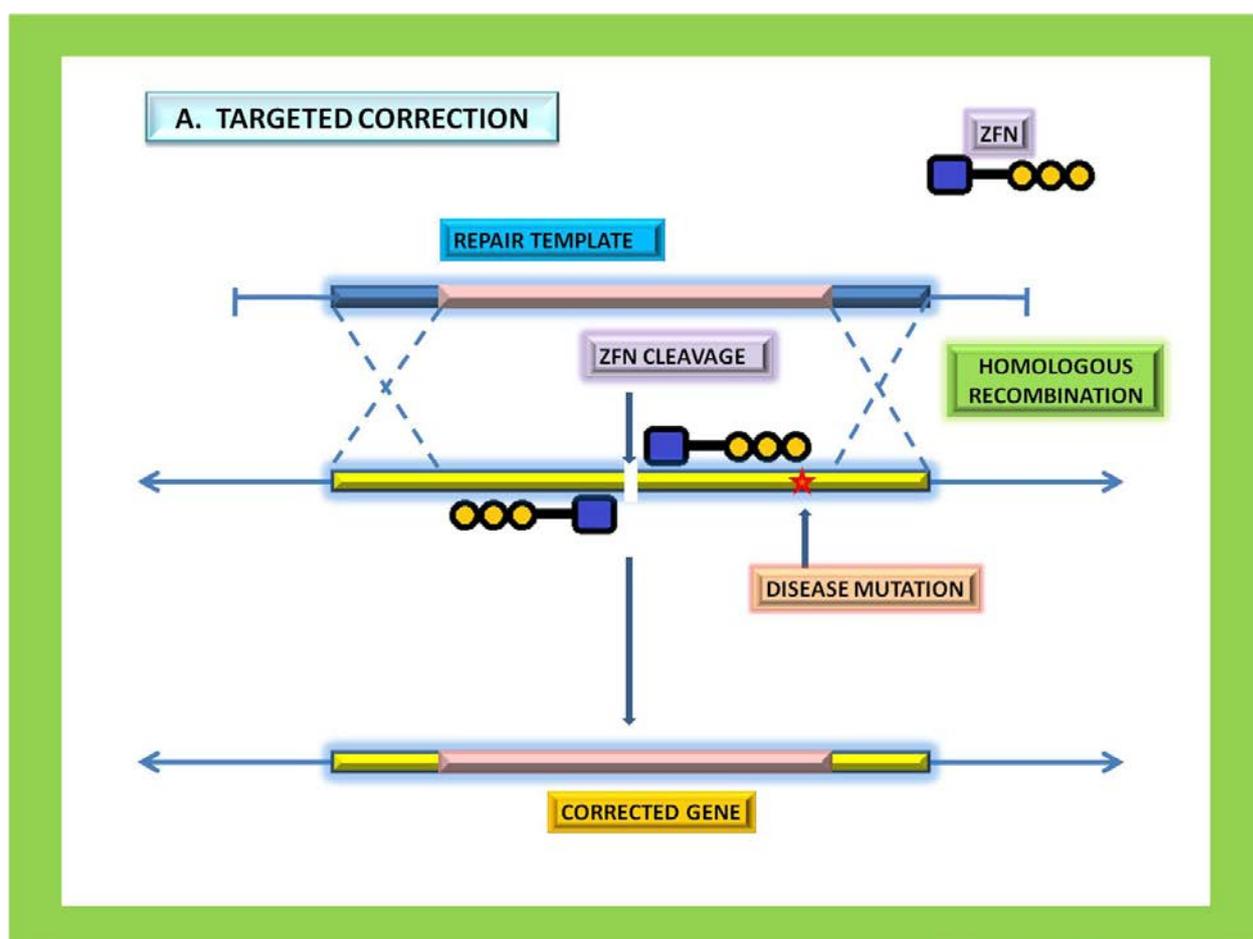
In the figure 6.1 expressed ZFNs pairs bind to and break the target genomic locus close to the disease-causing mutation. ZFNs comprise of chimeric zinc fingers (yellow) and *Fok1* endonuclease domains (blue). Further the repair template donor is used for the insertion of a DSB that stimulates homologous recombination.

Figure 6.2 Diagrammatic illustration of targeted gene addition strategy:
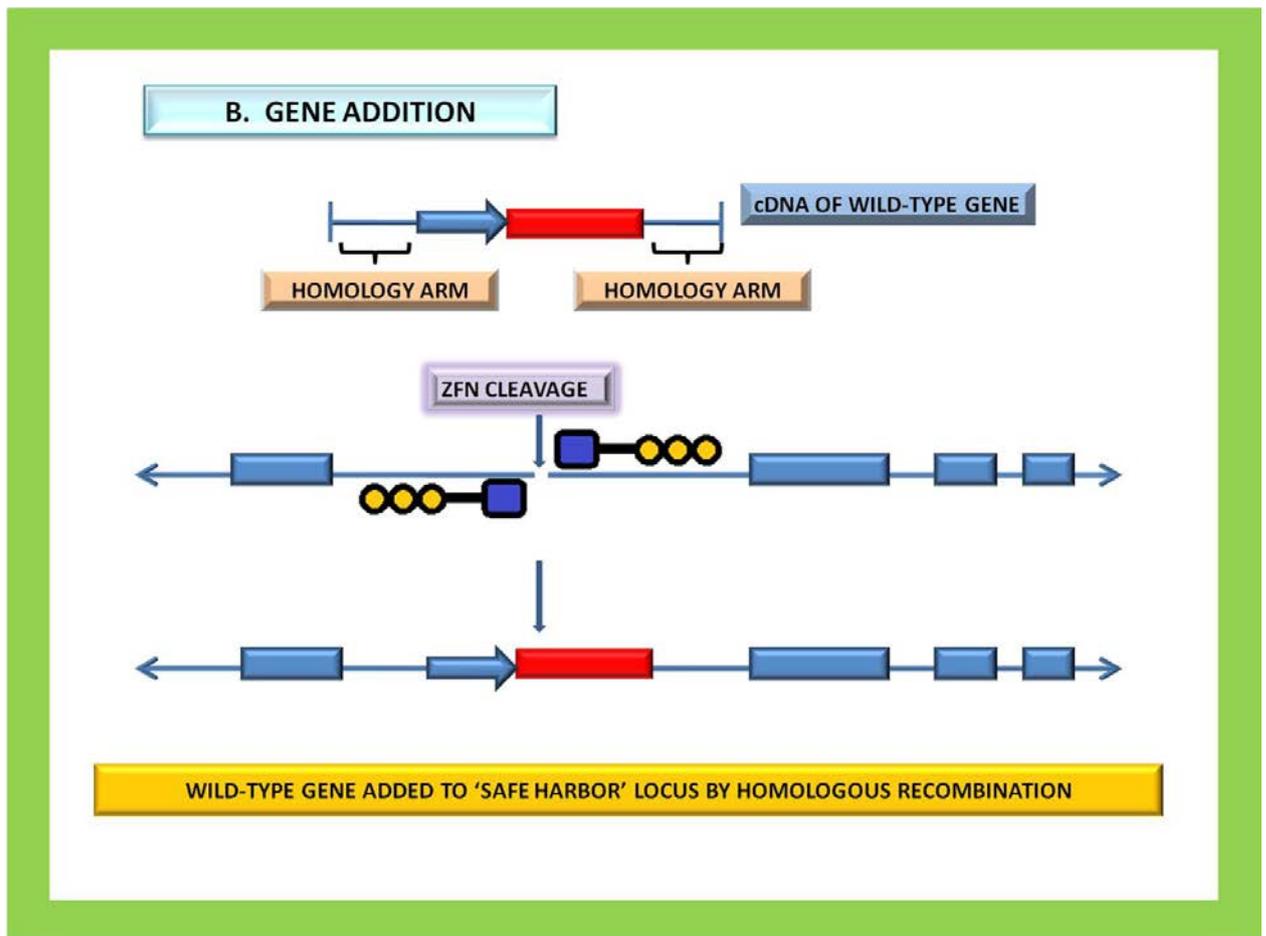
In the figure 6.2 that discusses targeted gene addition, the ZFN pair cleaves a predetermined 'safe harbor' locus. The expression cassette with a therapeutic gene (red) is bounded by homology arms to the safe harbor locus.