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IMPORTANCE AND APPLICATION OF GENE THERAPY

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ABSTRACT

Gene therapy was conceived as a treatment for monogenic (Mendelian) disease, hereditary diseases by complementation of a mutant gene with a normal gene. However, gene therapy also includes treatment of acquired human disease by delivery of DNA encoding a therapeutic protein, or introducing a fragment of nucleic acid to interrupt the mRNA of a pathogenic protein. A key component of the use of nucleic acids are the spectrum of strategies used to focus therapies on specific organs and/ or deliver genes to specific cells. Targeting is achieved through genetically engineered viruses, receptor–ligand interactions and antibodies. Therefore, these gene based reagents and the vehicles used to delivery them represent a novel form of “gene as drug”.

INTRODUCTION

Gene therapy is the insertion, alteration, or removal of genes within an individual's cells and biological tissues to treat disease. The most common form of gene therapy involves the insertion of functional genes into an unspecified genomic location in order to replace a mutated gene, but other forms involve directly correcting the mutation or modifying normal gene that enables a viral infection. Although the technology is still in its infancy, it has been used with some success. Scientific breakthroughs continue to move gene therapy toward mainstream medicine. Figure 1 show the gene therapy process. Scientists have taken the logical step of trying to introduce genes directly into human cells, focusing on diseases caused by single-gene defects, such as cystic fibrosis, haemophilia, muscular dystrophy and sickle cell anemia. However, this has proven more difficult than modifying bacteria, primarily because of the problems involved in carrying large sections of DNA and delivering them to the correct site on the gene. Today, most gene therapy studies are aimed at cancer and hereditary diseases linked to a genetic defect. Antisense therapy is not strictly a form of gene therapy, but is a related, genetically-mediated therapy. The most common form of genetic engineering involves the insertion of a functional gene at an unspecified location in the host genome. This is accomplished by isolating and copying the gene of interest, generating a construct containing all the genetic elements for correct expression, and then inserting this construct into a random location in the host organism. Other forms of genetic engineering include gene targeting and knocking out specific genes via engineered nucleases such as zinc finger nucleases, engineered I-CreI homing endonucleases, or nucleases generated from TAL effectors. An example of gene-knockout mediated gene therapy is the knockout of the human CCR5 gene in T-cells in order to control HIV infection. This approach is currently being used in several human clinical trials[1].

TYPES OF GENE THERAPY

Gene therapy may be classified into the two following types:

(A) Germ line gene therapy

In the case of germ line gene therapy, germ cells are modified by the introduction of functional genes, which are ordinarily integrated into their genomes. Therefore, the change due to therapy would be heritable. This new approach, theoretically, should be highly effective in counteracting genetic disorders, hereditary diseases. However, many jurisdictions prohibit this for application in human beings, at least for the present, for a variety of technical and ethical reasons.

(B) Somatic gene therapy

In the case of somatic gene therapy, the therapeutic genes are transferred into the somatic cells of a patient. Any modifications and effects will be restricted to the individual patient only, and will not be inherited by the patient's offspring or later generations.

Two basic gene therapy strategies have been investigated

1. In vivo gene therapy requires that the gene transfer vector be delivered in a cell-type selective manner, either through direct tissue injection, or perhaps someday, by receptor-mediated processes. In vivo gene therapy is done by targeting the gene delivery system to the (reconstructed liposomes or viruses). Importantly, neither of these gene therapy strategies involve reproductive germline desired cell type in the patient using either physical means such as tissue injection (brain tumor) or biolistics (dermal DNA vaccination), or potentially in the future, using systemic infusion of cell-specific receptor-mediated DNA carriers cells and therefore the genetic alteration will NOT be transmitted to the next generation. In many countries, human germline gene therapy is considered unethical. Figure 2 show in vivo gene therapy.

2. Ex-vivo gene therapy involves removing tissue from the patient, transfecting (or virally-infecting) the cells in culture, and then reimplanting the genetically altered cells to the patient. Ex-vivo gene therapy is performed by transfecting or infecting patient-derived cells in culture with vector DNA and then reimplanting the transfected cells into the patient. Two types of ex-vivo gene therapies under development are directed at fibroblasts, hematopoietic stem cells[2].

GENE DELIVERY

Gene delivery is unique in the sense, that it is the product of gene function, the protein, not the gene itself that is the therapeutic agent. Hence, we must not-only deliver the gene to its proper target but we must also assure that when the gene reaches its target it will arrive in a form that will produce the therapeutic agent in such a form that it too will be assured of reaching its specified target.

The steps involved in the delivery of the therapeutic agent include:

1. Administration of the nucleic acid in to the body which usually involves either in-vivo or in-vitro techniques.
2. Delivery of the nucleic acid from the site of administration to the nucleus which includes direction the bioavailability, uptake of the nucleic acid into the cell, and translocation of the nucleic acid from the cytosol to the nucleus.

3. Expression of the nucleic acid product including the normal steps in gene expression-transcription, translation, and post-translation modification. Expression is vital to the therapeutic efficacy of the gene in the sense that controlled expression of gene is important to proper biological function. With few exceptions delivery technologies result in continuous gene expression[3].

VECTORS IN GENE THERAPY

Viral vectors are a tool commonly used by molecular biologists to deliver genetic material into cells. This process can be performed inside a living organism (in vivo) or in cell culture (in vitro). Viruses have evolved specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. Delivery of genes by a virus is termed transduction and the infected cells are described as transduced. Molecular biologists first harnessed this machinery in the 1970s. Paul Berg used a modified SV40 virus containing DNA from the bacteriophage lambda to infect monkey kidney cells maintained in culture.

Key properties of a viral vector

Viral vectors are tailored to their specific applications but generally share a few key properties.

- Safety: Although viral vectors are occasionally created from pathogenic viruses, they are modified in such a way as to minimize the risk of handling them. This usually involves the deletion of a part of the viral genome critical for viral replication. Such a virus can efficiently infect cells but, once the infection has taken place, requires a helper virus to provide the missing proteins for production of new virions.
- Low toxicity: The viral vector should have a minimal effect on the physiology of the cell it infects.
- Stability: Some viruses are genetically unstable and can rapidly rearrange their genomes. This is detrimental to predictability and reproducibility of the work conducted using a viral vector and is avoided in their design.
- Cell type specificity: Most viral vectors are engineered to infect as wide a range of cell types as possible. However, sometimes the opposite is preferred. The viral receptor can be modified to target the virus to a specific kind of cell.
- Identification: Viral vectors are often given certain genes that help identify which cells took up the viral genes. These genes are called Markers; a common marker is antibiotic resistance

to a certain antibiotic. The cells can then be isolated easily as those that have not taken up the viral vector genes do not have antibiotic resistance and so cannot grow in a culture with antibiotics present.

Retroviruses

Retroviruses are one of the mainstays of current gene therapy approaches. The recombinant retroviruses such as the Maloney murine leukemia virus have the ability to integrate into the host genome in a stable fashion. They contain a reverse transcriptase that allows integration into the host genome. They have been used in a number of FDA-approved clinical trials such as the SCID-X1 trial. Retroviral vectors can either be replication-competent or replication-defective. Replication-defective vectors are the most common choice in studies because the viruses have had the coding regions for the genes necessary for additional rounds of virion replication and packaging replaced with other genes, or deleted. These viruses are capable of infecting their target cells and delivering their viral payload, but then fail to continue the typical lytic pathway that leads to cell lyses and death. Conversely, replication-competent viral vectors contain all necessary genes for virion synthesis, and continue to propagate themselves once infection occurs. Because the viral genome for these vectors is much lengthier, the length of the actual inserted gene of interest is limited compared to the possible length of the insert for replication-defective vectors. Depending on the viral vector, the typical maximum length of an allowable DNA insert in a replication-defective viral vector is usually about 8-10 kB. While this limits the introduction of many genomic sequences, most cDNA sequences can still be accommodated. The primary drawback to use of retroviruses such as the Moloney retrovirus involves the requirement for cells to be actively dividing for transduction. As a result, cells such as neurons are very resistant to infection and transduction by retroviruses. There is concern that insertional mutagenesis due to integration into the host genome might lead to cancer or leukemia[4].

Adenoviruses

As opposed to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division. This limits their use in basic research, although adenoviral vectors are occasionally used in invitro experiments. Their primary applications are in gene therapy and vaccination. Since humans commonly come in contact with adenoviruses, which cause respiratory, gastrointestinal and eye infections, they trigger a rapid immune response with

potentially dangerous consequences. To overcome this problem scientists are currently investigating adenoviruses to which humans do not have immunity.

NON-VIRAL METHODS

Non-viral methods present certain advantages over viral methods, with simple large scale production and low host immunogenicity being just two. Previously, low levels of transfection and expression of the gene held non-viral methods at a disadvantage; however, recent advances in vector technology have yielded molecules and techniques with transfection efficiencies similar to those of viruses.

Injection of Naked DNA

This is the simplest method of non-viral transfection. Clinical trials carried out of intramuscular injection of a naked DNA plasmid have occurred with some success; however, the expression has been very low in comparison to other methods of transfection. In addition to trials with plasmids, there have been trials with naked PCR product, which have had similar or greater success. Cellular uptake of naked DNA is generally inefficient. Research efforts focusing on improving the efficiency of naked DNA uptake have yielded several novel methods, such as electroporation, sonoporation, and the use of a "gene gun", which shoots DNA coated gold particles into the cell using high pressure gas[5].

PHYSICAL METHODS TO ENHANCE DELIVERY

Electroporation

Electorporation is a method that uses short pulses of high voltage to carry DNA across the cell membrane. This shock is thought to cause temporary formation of pores in the cell membrane, allowing DNA molecules to pass through. Electroporation is generally efficient and works across a broad range of cell types. However, a high rate of cell death following electroporation has limited its use, including clinical applications.

More recently a newer method of electroporation, termed electron-avalanche transfection, has been used in gene therapy experiments. By using a high-voltage plasma discharge, DNA was efficiently delivered following very short (microsecond) pulses. Compared to electroporation, the technique resulted in greatly increased efficiency and less cellular damage.

Gene Gun

The use of particle bombardment, or the gene gun, is another physical method of DNA transfection. In this technique, DNA is coated with gold particles and loaded into a device which generates a force to achieve penetration of DNA/gold into the cells.

Sonoporation

Sonoporation uses ultrasonic frequencies to deliver DNA into cells. The process of acoustic cavitation is thought to disrupt the cell membrane and allow DNA to move into cells.

Magnetofection

In a method termed magnetofection, DNA is complexed to magnetic particles, and a magnet is placed underneath the tissue culture dish to bring DNA complexes into contact with a cell monolayer.

CHEMICAL METHODS TO ENHANCE DELIVERY

Oligonucleotides

The use of synthetic oligonucleotides in gene therapy is to inactivate the genes involved in the disease process. There are several methods by which this is achieved. One strategy uses antisense specific to the target gene to disrupt the transcription of the faulty gene. Another uses small molecules of RNA called siRNA to signal the cell to cleave specific unique sequences in the mRNA transcript of the faulty gene, disrupting translation of the faulty mRNA, and therefore expression of the gene. A further strategy uses double stranded oligodeoxynucleotides as a decoy for the transcription factors that are required to activate the transcription of the target gene. The transcription factors bind to the decoys instead of the promoter of the faulty gene, which reduces the transcription of the target gene, lowering expression. Additionally, single stranded DNA oligonucleotides have been used to direct a single base change within a mutant gene. The oligonucleotide is designed to anneal with complementarity to the target gene with the exception of a central base, the target base, which serves as the template base for repair. This technique is referred to as oligonucleotide mediated gene repair, targeted gene repair, or targeted nucleotide alteration.

Lipoplexes and polyplexes

To improve the delivery of the new DNA into the cell, the DNA must be protected from damage and its entry into the cell must be facilitated. To these end new molecules, lipoplexes and

polyplexes, have been created that have the ability to protect the DNA from undesirable degradation during the transfection process. Plasmid DNA can be covered with lipids in an organized structure like a micelle or a liposome. When the organized structure is complexed with DNA it is called a lipoplex. There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged). Initially, anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors. However, in spite of the facts that there is little toxicity associated with them, that they are compatible with body fluids and that there was a possibility of adapting them to be tissue specific; they are complicated and time consuming to produce so attention was turned to the cationic versions.

Cationic lipids, due to their positive charge, were first used to condense negatively charged DNA molecules so as to facilitate the encapsulation of DNA into liposomes. Later it was found that the use of cationic lipids significantly enhanced the stability of lipoplexes. Also as a result of their charge, cationic liposomes interact with the cell membrane, endocytosis was widely believed as the major route by which cells uptake lipoplexes. Endosomes are formed as the results of endocytosis, however, if genes can not be released into cytoplasm by breaking the membrane of endosome, they will be sent to lysosomes where all DNA will be destroyed before they could achieve their functions. It was also found that although cationic lipids themselves could condense and encapsulate DNA into liposomes, the transfection efficiency is very low due to the lack of ability in terms of “endosomal escaping”. However, when helper lipids (usually electroneutral lipids, such as DOPE) were added to form lipoplexes, much higher transfection efficiency was observed. Later on, it was figured out that certain lipids have the ability to destabilize endosomal membranes so as to facilitate the escape of DNA from endosome, therefore those lipids are called fusogenic lipids. Although cationic liposomes have been widely used as an alternative for gene delivery vectors, a dose dependent toxicity of cationic lipids were also observed which could limit their therapeutic usages. The most common use of lipoplexes has been in gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell and decrease the activity of oncogenes. Recent studies have shown lipoplexes to be useful in transfecting respiratory epithelial cells, so they may be used for treatment of genetic respiratory diseases such as cystic fibrosis. Complexes of polymers with DNA are called polyplexes. Most polyplexes consist of cationic polymers and their production is regulated by

ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm, so to this end, co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis, the process by which the polyplex enters the cell) such as inactivated adenovirus must occur. However, this isn't always the case, polymers such as polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan.

Dendrimers

A dendrimer is a highly branched macromolecule with a spherical shape. The surface of the particle may be functionalized in many ways and many of the properties of the resulting construct are determined by its surface. In particular it is possible to construct a cationic dendrimer, i.e. one with a positive surface charge. When in the presence of genetic material such as DNA or RNA, charge complimentarity leads to a temporary association of the nucleic acid with the cationic dendrimer. On reaching its destination the dendrimer-nucleic acid complex is then taken into the cell via endocytosis. In recent years the benchmark for transfection agents has been cationic lipids. Limitations of these competing reagents have been reported to include: the lack of ability to transfect a number of cell types, the lack of robust active targeting capabilities, incompatibility with animal models, and toxicity. Dendrimers offer robust covalent construction and extreme control over molecule structure, and therefore size. Together these give compelling advantages compared to existing approaches. Producing dendrimers has historically been a slow and expensive process consisting of numerous slow reactions, an obstacle that severely curtailed their commercial development, a process that not only reduced cost by a magnitude of three, but also cut reaction time from over a month to several days. These new "Priostar" dendrimers can be specifically constructed to carry a DNA or RNA payload that transfects cells at a high efficiency with little or no toxicity.[6-8]

HYBRID METHODS

There have been some hybrid methods developed that combine two or more techniques. Virosomes are one example; they combine liposomes with an inactivated HIV or influenza virus. This has been shown to have more efficient gene transfer in respiratory epithelial cells than either viral or liposomal methods alone. Other methods involve mixing other viral vectors with cationic lipids or hybridising viruses.

PROBLEMS AND ETHICS

Some of the problems of gene therapy include

- Short-lived nature of gene therapy – Before gene therapy can become a permanent cure for any condition, the therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving any long-term benefits. Patients will have to undergo multiple rounds of gene therapy.
- Immune response – Anytime a foreign object is introduced into human tissues, the immune system has evolved to attack the invader. The risk of stimulating the immune system in a way that reduces gene therapy effectiveness is always a possibility. Furthermore, the immune system's enhanced response to invaders that it has seen before makes it difficult for gene therapy to be repeated in patients.
- Problems with viral vectors – Viruses, the carrier of choice in most gene therapy studies, present a variety of potential problems to the patient —toxicity, immune and inflammatory responses, and gene control and targeting issues. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease.
- Multigene disorders – Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy.
- Chance of inducing a tumor (insertional mutagenesis) - If the DNA is integrated in the wrong place in the genome, for example in a tumor suppressor gene, it could induce a tumor. This has occurred in clinical trials for X-linked severe combined immunodeficiency (X-SCID) patients, in which hematopoietic stem cells were transduced with a corrective transgene using a retrovirus, and this led to the development of T cell leukemia in 3 of 20 patients[9].

APPLICATIONS[10-15]

Over 600 clinical gene therapy trials using various strategies for treatment of hereditary and acquired disease are underway with many other protocols in development. Clinical trials fall into such general categories as genetics diseases, cancer, infectious diseases, and other multifactorial acquired diseases. Such as arthritis or vascular disease.

Table 1. Human gene transfer studies in which the transferred gene may have produce a biologically relevant response.

Disease	Gene transfer method	Gene product	Target cells	Reported biological response
Adenosine deaminase deficiency	Retro virus Ex vivo	Adenosine deaminase	Blood T cells,cord blood CD34+ stem cells	Partial increase in immune response
Cystic fibrosis	Adenovirus In vivo	CFTR	Nasal epithelium	Partial correction of epithelial abnormalties
Cystic fibrosis	Plasmidliposome In vivo	CFTR	Nasal epithelium	Partial correction of epithelial abnormalties
Familial Hypercholesterolemia	Retro virus Ex vivo	Low density lipoprotein receptor	Hepatocytes	Partial correction of Lipid abnormalities
Malignant tumors	Plasmidliposome In vivo	HLA-B7 and beta-2	Melanoma,colorectal carcinoma and renal cell carcinoma	Immune response to tumor
Malignant tumors	Retro virus Ex vivo	Interleukin-4	Fibroblast used as vaccine	Immune response to tumor
Malignant tumors	Retro virus Ex vivo	Interleukin-2	Neuroblastoma used as a vaccine	Immune response to tumor

GENE-THERAPY IN CANCER

A number of cancers can be relatrd germline mutations of particular genes,breast cancer being a particularly good example where,the majority of mutations causing cancer are somatic cell mutations. Mutations in protooncogenes.and tumor suppresor genes are major cause of cancer.oncogenes were first discovered in tumor cellsbut are also found in normal cells where

they are termed Protooncogenes. The protooncogene product functions as a regulator of cell growth and differentiation where it is involved in the transmission of external cellular environment growth stimuli to the cell apparatus inside the nucleus controlling cell growth. The transformation of protooncogene may occur via the following molecular mechanism-

1) Point mutation of the protooncogene,altering the structure of the gene product such that it becomes a more efficient or unregulated protein.

2) Amplification of the protooncogene through production of multiple independent single gene copies.

Oncogenes produce uncontrolled growth through-

1) Excess production of growth factor.

2) Protein kinase abnormally altering cell-regulation proteins through phosphorylation.

3) Abnormal cell regulation protein signaling growth.

4) Altered transcription factors stimulating cell replication.

Table 2. Functional classification of selected oncogene and associated human tumors

FUNCTION	ONCOGENE	ASSOCIATED TUMORS
Growth factor	HST KS3	Gastric cancer Kaposi sarcoma
Growth factor receptor	NEU/ERB-B2 ERB-B TRK	Breast,ovary,gasric cancer Breast cancer,glioblastoma Colon cancer
Signal transducing proteins	Ha-RAS N-RAS GSP	Bladder cancer Leukemias Pituitary tumors
Protein kinases	RAF ABL	Gastric cancer Leukemia
Nuclear transcription factor	N-MYC L-MYC	Neuroblastoma Small cell lung cancer
Membrane proteins	MAS RET	Breast cancer Papillary thyroid cancer

Tumor suppressor genes are also normal components of cell regulation normally the suppressor gene products will arrest replication of a cell with damaged DNA until the DNA is repaired. Failure to repair the DNA and resume normal function will result in programmed cell death (APOPTOSIS). Therefore ,the loss of suppressor gene may result in uncontrolled cell growth

arising from cell transformation .Gene therapy can be carried out in which the tumor suppressor gene is inserted in to tumor cell lines to stimulate apoptosis killing the tumor cells.

Table 3. Tumor suppressor genes

GENE	GENE PRODUCT	TUMOR ASSOCIATION
RB(13q)	110-kDa nuclear hypophosphorylated protein,negative cell-cycle regulator	Retinoblastoma Osteosarcoma Small cell lung cancer Breast cancer
P53(17p)	53-kDa sequence specific DNA-binding protein and transcriptional activator	Most common alteration in human cancer
DCC(18q)	1447 amino acid Transmembrane protein with homology to known adhesion molecules ,role in terminal cell differentiation	Colorectal cancer
MTSI(9q21)	148 aminoacid protein inhibitor of cycline dependent kinase-4	Familial melanoma
BRCA2(13q)	Possibly brush-1	Familial breast cancer
NF-1	Neurofibromin,probably nagative reguletor for p21 ras	Von recklinghausens Neurofibromatosis
NF-2	Schwannomin,regulator of Cellular response to External environment	Neurofibromatosis type-2
WT-1	50-kda gene related to the early growth response gene	Wilms tumor
VHL	Protein with short homology to a Glycan-anchored memberane protein to t.brucie	Renal cell cancer Pancreatic cancer

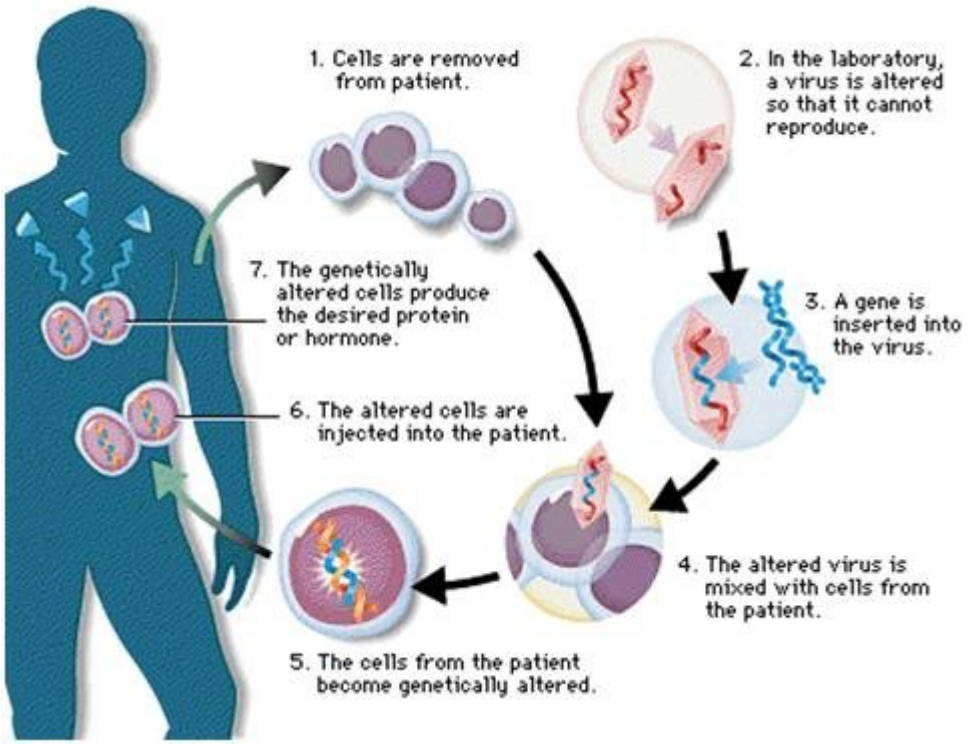

Figure 1: Gene-Therapy Process

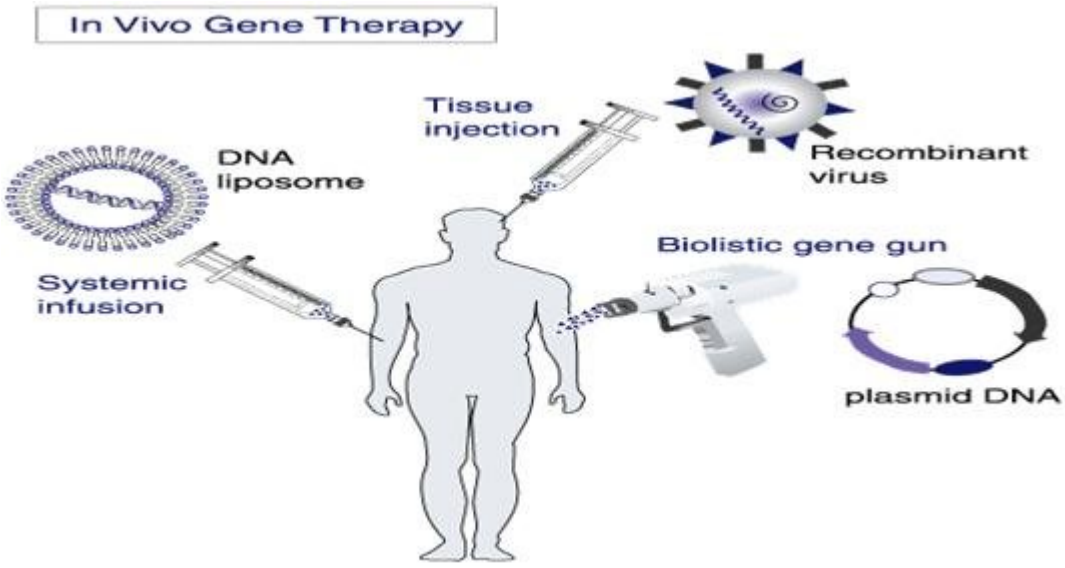

Figure 2: In vivo Gene Therapy

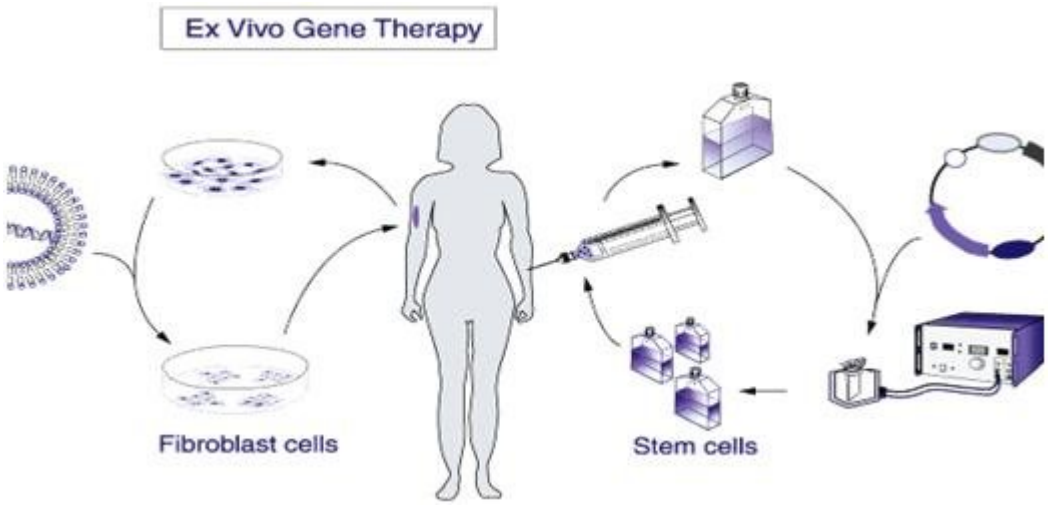

Figure 3: Ex-vivo Gene Therapy

CONCLUSION

Gene therapy was originally conceived as a treatment for monogenic disease by complementation of mutant gene with normal gene. Genetic technology has evolved over the past 30 years to the stage where we are on the verge of determining the entire sequence of the 3 billion base pairs in the human genome. The human genome project (HGP) is developing scientific technology for the manipulation & analysis of the human genome. This analysis will in turn provide valuable information on the relationship between genotype & potential for serious genetic disease. Gene therapy is being considered as a potential medical revolution. Initially, it was viewed as an approach for treating hereditary diseases, but now wide recognition of its potential role in the treatment of acquired diseases such as cancer is being envisaged. The future will see an altered form of health care utilizing a genetic information infrastructure to contain costs and predict outcomes, create advanced personalized therapies, and develop a predict-and-manage paradigm of healthcare. Despite many potential advantages of non-viral retinal gene therapy, success has been limited to date and many studies have not been independently replicated. It remains difficult to target photoreceptor cells and to achieve high and persistent trans gene expression without causing harm to the sensitive neuronal tissue by using physical or chemical transfections methods. To better estimate its effectiveness, non-viral retinal gene therapy should ideally be tested against viral vectors in future studies. Future developments may include new non-viral biological gene delivery vehicles that have so far been unexplored for retinal gene therapy.50 For instance, exosomes have been targeted to neurons in the brain;51

however, their current loading capacity for oligo nucleotides would need to be improved for delivery of complete gene coding sequences. The future concept of using DNA plasmid-based approaches instead of viral vectors for retinal gene therapy is very appealing, but still long way off.

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