GENE THERAPY, PHYSIOLOGICAL APPLICATIONS, PROBLEMS AND PROSPECTS - A REVIEW

[1]UGWU, Godwin Chigozie, [1]EGBUJI, Jude Victor Ifeanyi, [1]OKANYA, Laureta Chinagorom, [2]OMEJE, Joy Nwamaka and [1]EYO, Joseph Effiong

[1]Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria.

[2]Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

Corresponding Author: Ugwu, G. C. Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria. **Email:** godwin.ugwu@unn.edu.ng **Phone:** +234 8064005944

Received: June 16, 2017 **Revised:** May 23, 2018 **Accepted**: July 19, 2019

ABSTRACT

Gene therapy can be defined as the use of DNA as a pharmaceutical agent to treat disease. It is also an experimental medical treatment that manipulates a gene or genes within cells in order to produce proteins that change the function of those cells. The physiological applications, problems and prospects of gene therapy are reviewed in this study. The different types of gene therapy such as germline gene therapy, somatic gene therapy and chimeraplasty gene therapy are discussed. Polymerase chain reaction (PCR), nanoparticles, sonoporation, electroporation and gene gun are the techniques used in gene therapy. Polymerase chain reaction (PCR) is used in medical and biological research. Nanoparticles have been widely used in the field of drug and gene delivery to target cells. Sonoporation allows uptake of large molecules of DNA into the cell, in a process called cell transformation. Electroporation is highly efficient for the introduction of foreign genes in tissue culture cells, in tumor treatment and cell-based therapy. A gene gun is a device for injecting cells with genetic information to plant cells. Gene therapy is applied in medicine, agriculture, loss and gain of function, tracking and expression studies. Some problems bedeviling gene therapy include insertional mutagenesis, mutagenic disorders, problem of viral vectors, immune response etc. Gene therapy has the potential to eliminate and prevent hereditary diseases such as cystic fibrosis and is a possible cure for Alziehmer's disease and cancer, enhance agricultural productivity of farm animals, and in the production of genetically modified animals (GMOs) which will further help in medical and biomedical research.

Keywords: Gene therapy, Chimeraplasty, Nanoparticles, Sonoporation, Electroporation, Gene gun, Polymerase chain reaction

INTRODUCTION

In gene therapy, DNA is use as a pharmaceutical agent to treat disease. It is also an experimental medical treatment that manipulates a gene or genes within cells in order to produce proteins that change the function of those cells (Alberts *et al.,* 2002). It derives its name from the idea that DNA can be used to supplement or alter genes within an individual cells. The most common form of gene therapy involves using DNA strands that encode functional therapeutic gene in order to replace a mutated gene. In gene therapy, DNA that encodes a therapeutic protein is packaged within a "vector" which is used to get the DNA

inside the cells within the body. The DNA now inside becomes expressed by the cell machinery resulting in the production of a therapeutic protein which in turn treats the patient's diseases (Brivanlou and Darnell, 2002).

Gene therapy was first conceptualized as a result of efforts to treat and cure some known genetic disorders, most of which lack an effective therapy. The original goal of gene therapy was to substitute a healthy gene for a defective one, or to repair a faulty gene, thereby dominating symptoms of the disease or defect (Cole-Strauss *et al.,* 1996). However, researchers have moved beyond inherited genetic disorders to treat other kinds of diseases, with nearly 75 % of all clinical trials aimed at treatments for cancer, acquired immunodeficiency syndrome (AIDS), Alzheimer's disease, diabetes mellitus and arthritis, all of which involve genetic susceptibility to illness.

Although gene therapy offers seemingly limitless possibilities, researchers have been thwarted by many technical problems. Most clinical trials of gene therapy have not resulted in enough improvement in the patients underlying condition to consider it an unqualified success and to justify treating large numbers of people (Coghlan, 1999).

The extraordinary potential of gene therapy had also raised alarms among critics who warn that the technology could go too far. They note that gene therapy could offer wealthy families opportunities for genetic enhancement unavailable to the poor. More troubling still for some critics is gene therapy's potential to narrow the human gene pool, producing unknown and possibly harmful consequences (Cheng *et al.,* 1994).

Overview of Gene Therapy: A gene is a long segment of the molecule deoxyribonucleic acid (DNA). This segment, composed of minute subunits called nucleotide bases, serves as the blueprint for manufacturing a single protein or enzyme needed for the structure or function of cells (Griffiths *et al.,* 2000). In humans, genes are compressed and bundled into a set of 23 pairs of chromosomes which stabilize and protect the DNA. Any tiny error in the arrangement of a genes nucleotide bases can lead to the production of a protein or enzyme that functions improperly, disrupting cellular biological activities such as codons for proteins or enzymes production (Isenbarger *et al.,* 2008). Genes correspond to regions within DNA which is composed of a chain of four different types of nucleotides: adenine, guanine, thymine and cytosine. The sequence of these nucleotides is the genetic information organisms inherit DNA naturally occurs in a double stranded from, with the nucleotide base pairs on each strand complementary to each other. Each strand can act as a template for creating a new partner strand. This is the physical method for making copies of genes that can be inherited. Viruses on the other hand use a similar molecule ribonucleic acid, RNA, instead of DNA as their genetic material (Hershey and Chase, 1952).

The nucleotides sequence in a gene is translated by cells to produce a chain of amino acids; the order of amino acids in a protein corresponds to the order of nucleotides in the gene. The relationship between nucleotide sequence and amino acids is known as the genetic code. The types of amino acids in a protein determine how it folds into a three-dimensional shape. This structure is in turn responsible for the protein's function. A change to the DNA in a gene can change a protein's amino acid changing its shape and function; this can have a dramatic effect on the whole organism (Gura, 1999).

Gene Expression: Genes generally express their functional effect through the production of proteins which are complex molecules responsible for most functions in the cells. Proteins are chains of amino acids, and the DNA sequence of a gene (through an RNA intermediate) is used to produce a specific protein sequence (Mayeux, 2005). The process called transcription begins with the production of an RNA molecule with a sequence that matches the gene's DNA sequence. The messenger RNA (mRNA) molecule is then used to produce a corresponding amino acid sequence through a process called translations. Each group of three nucleotides in the sequence called a codon corresponds either to one of the twenty possible amino acids in a protein or an

instruction to end the amino acid sequence and this corresponds to the genetic code. The flow of information is unidirectional; a situation in which information is transferred from nucleotide sequences into the amino acid sequence of proteins, but it never transfers from protein back into the sequence of DNA (Lodish *et al.,* 2000). A single nucleotide difference within DNA can cause a change in the amino acid sequence of a protein. Because protein structures are the result of their amino acid sequences, some changes can dramatically change the properties of a protein by destabilizing the structure or changing the surface of the protein in a way that changes its interaction with other proteins and molecules (Saiki *et al.,* 1985). For example, sickle cell anaemia is a human genetic disease that results from a single base difference within the coding region for the β-globin section of hemoglobin, causing a single amino acid change that changes hemoglobin's physical properties. Sickle-cell versions of hemoglobin stick to themselves, stacking to form fibers that distort the shape of red blood cells carrying the protein. These sickle-shaped cells no longer flow smoothly through blood vessels, having a tendency to clog or degrade, causing the medical problems associated with this disease (Nabel *et al.,* 1992; Cole-Strauss *et al.,* 1996). Some genes are transcribed into RNA but are not translated into protein products, such RNA molecules are called non-coding RNA. In some cases, these products fold into structures which are involved in critical cell functions (e.g. ribosomal RNA and transfer RNA). RNA can also have regulatory effect through hybridization interactions with other RNA molecules (e.g. micro RNA) (Sails, 2004).

Gene Regulation: The genome of a given organism contains thousands of genes, but not all these genes need to be active at any given moment. A gene is expressed when it is being transcribed into mRNA (and translated into protein), and there exist many cellular methods of controlling the expression of genes such that proteins are produced only when needed by the cell. Transcription factors are regulatory proteins that bind to the start of genes, either promoting or inhibiting the transcription of the gene (Wolf *et al.,* 2002). Within the genome of *Escherichia coli* bacteria, for example, there exists a series of genes necessary for the synthesis of the amino acid tryptophan. Once tryptophan is already available in the cell, the genes for tryptophan synthesis are no longer needed. The presence of tryptophan directly affects the activity of the genes. Tryptophan molecules bind to the tryptophan repressor (a transcription factor), changing the repressor's structure such that the repressor binds to the genes. The tryptophan repressor blocks the transcription and expression of the genes, thereby creating negative feedback regulation of the tryptophan synthesis process (Yang, 2007).

Differences in gene expression are especially clear within multicellular organisms where cells all contain the same genome but have very different structures and behaviors due to the expression of different sets of genes. All the cells in a multicellular organism derive from a single cell, differentiating into variant cell types in response to external and intercellular signals and gradually establishing different patterns of gene expression to create different behaviors. As no single gene is responsible for the development of structures within multicellular organisms, these patterns arise from the complex interactions between many cells (Yoon *et al.,* 1996).

Within eukaryotes there exist structural features of chromatin that influence the transcription of genes, often in the form of modifications to DNA and chromatin that are stably inherited by daughter cells. These features are called "epigenetic" because they exist "on top" of the DNA sequence and retain inheritance from one cell generation to the next. These epigenetic features will cause different cell types grown within the same medium to retain very different properties. Although epigenetic features are generally dynamic over the course of development, some, like the phenomenon of paramutation, have multigenerational inheritance and exist as rare exceptions to the general rule of DNA as the basis for inheritance (Pathak *et al.,* 2009).

Mutations: During the process of DNA replication, errors occasionally occur in the

polymerization of the second strand. These errors, called mutations, can have an impact on the phenotype of an organism, especially if they occur within the protein coding sequence of a gene (Alberts *et al.,* 2002). Error rates are usually very low. One error in every 10 – 100 million bases due to the "proofreading" ability of DNA polymerases (without proofreading error rates are a thousand fold higher; because many viruses rely on DNA and RNA polymerases that lack proofreading ability, they experience higher mutation rates). Processes that increase the rate of changes in DNA are called mutagenic: mutagenic chemicals promote errors in DNA replication, often by interfering with the structure of base-pairing, while UV radiation induces mutations by causing damage to the DNA structure. Chemical damage to DNA occurs naturally as well, and cells use DNA repair mechanisms to repair mismatches and breaks in DNA. Nevertheless, the repair sometimes fails to return the DNA to its original sequence (Berg *et al*., 2002). In organisms that use chromosomal crossover to exchange DNA and recombine genes, errors in alignment during meiosis can also cause mutations. Errors in crossover are especially likely when similar sequences cause partner chromosomes to adopt a mistaken alignment; this makes some regions in genomes more prone to mutating in this way (Cheng *et al*., 1994). These errors create large structural changes in DNA sequence such as duplications, inversions, or deletions of entire regions, or the accidental exchanging of whole parts between different chromosomes (called translocation) (Newton *et al.,* 1989).

Mutations alter an organism's genotype and occasionally this causes different phenotypes to appear. Most mutations have little effect on an organism's phenotype, health, or reproductive fitness. Mutations that do have an effect are usually deleterious, but occasionally some can be beneficial. Studies in the fly *Drosophila melanogaster* suggest that if a mutation changes a protein produced by a gene, about 70 percent of these mutations will be harmful with the remainder being either neutral or weakly beneficial (Felgner and Ringold, 1989). Over many generations, the genomes of organisms can change significantly, resulting in the phenomenon of evolution. Selection for beneficial mutations can cause a species to evolve into forms better able to survive in their environment, a process called adaptation (Griffiths *et al.,* 2000). New species are formed through the process of speciation, often caused by geographical separations that prevent populations from exchanging genes with each other. The application of genetic principles to the study of population biology and evolution is referred to as the modern synthesis (Imes *et al.,* 2006).

By comparing the homology between different species' genomes it is possible to calculate the evolutionary distance between them and when they may have diverged (called a molecular clock). Genetic comparisons are generally considered a more accurate method of characterizing the relatedness between species than the comparison of phenotypic characteristics (Jaenisch and Bird, 2003). The evolutionary distances between species can be used to form evolutionary trees; these trees represent the common descent and divergence of species over time, although they do not show the transfer of genetic material between unrelated species known as horizontal gene transfer and most common in bacteria (Gura, 1999).

MATERIALS AND METHODS

A comprehensive internet search of literature on gene therapy was undertaken using Google search. Literatures recovered were analyzed in pros and relevant cited tables and figures adopted.

RESULT AND DISCUSSION

Types of Gene Therapy

1. Germline Gene Therapy: Germline gene therapy involves the modification of germ cells (gametes) that will pass the change on to the next generation. With germline therapy, genes sequence can be corrected in the egg or the in sperm that is being used to conceive. The child that results would be spared certain genetic problems that might otherwise have occurred

(Cole-Strauss *et al.,* 1999). In living organisms, every cell descends from the fertilized egg, thus every cell in the offspring will possess transplanted gene. This would be a far more effective way of transferring genes than the ones presently used in somatic cell therapies, where genes into the cells of children or adults usually enter only a small portion of the person's cells and eventually stop functioning (Cheng *et al.,* 1994).

The technology of gene therapy is based on the effective delivery of the corrective genes and to do this, scientists have developed gene delivery vehicles called vectors. These vectors encapsulate therapeutic genes for delivery into the targeted cells. Many of the vectors currently in use are based on attenuated or modified versions of viruses. Plasmids, which are circular pieces of DNA extracted from bacteria (Figure 1), are also used as vectors (Gura, 1999).

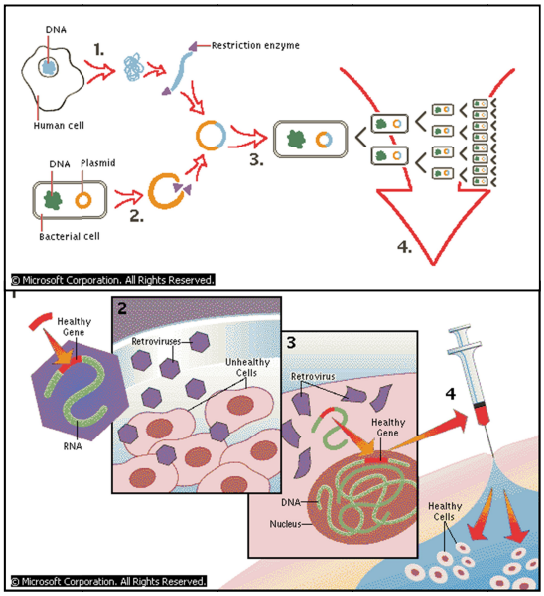

Figure 1: Mechanism of gene delivery. Source: Gura (1999)

The therapeutic gene to be transferred is extracted from the cell of a healthy individual. The gene is extracted by cutting the DNA using a restriction enzyme (restriction enzymes "digest" DNA at designated nucleotide locations along the DNA chain). There are different types of restriction enzymes, each being specific to the location of the DNA chain that it will cut. The section of cut DNA has to be intact then a technique called electrophoresis is used to separate the selected pieces of DNA and remove the genes that contained the DNA sequence that coded for the polypeptides needed (Jaroslav *et al.,* 2002).

Similar restriction enzymes are used to remove a section of enzyme from a plasmid or virus and using ligase (a special enzyme used to "glue" a foreign piece of DNA into a donor cell) the therapeutic DNA sequence is placed in the DNA of the vector. In the case of a virus, instead of causing illness the virus carries the normal genes into the target cells where they begin functioning (Lawyer *et al.,* 1993).

Germ line therapy which is the focus of this presentation is technically more difficult and raises many ethical challenges. The two main methods of performing germline therapy would be: i) To treat a pre-embryo that carries a serious genetic defect before implantation into the mother (this requires the use of in vitro fertilization techniques); or ii) to treat the germ cells (sperm or egg cells) of the afflicted adults so that their genetic defects would not be passed on to their offspring (Figure 2). This approach requires the technical expertise to remove the defective genes and insert a properly functioning replacement (Khan *et al.,* 2008).

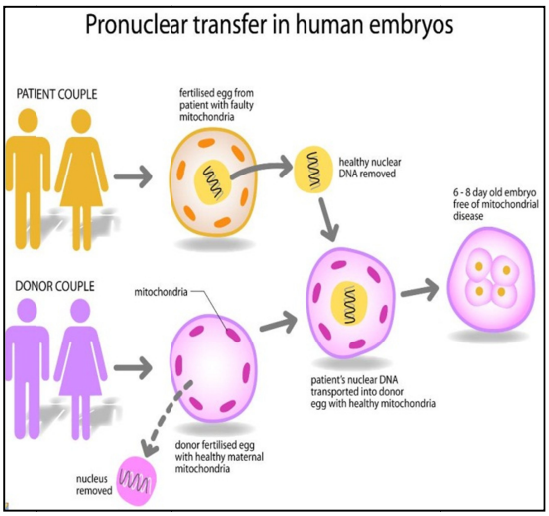

Figure 2: Illustration showing germline gene therapy involving pronuclear transfer in human embryos to prevent diseases. Source: Australian Science and Media Centre (2010)

Germline gene therapy is advancing at a rapid pace, so fast that new technologies are being readied for use without answering concerns about safety. The safety concern of this type of engineering arises because of a lack of knowledge about the human genome. It is quite possible that a valuable gene could be erased (Saiki *et al.,* 1985). However, only having one copy of the gene confers added resistance to malaria, making the sickle cell gene beneficial to people living in regions where malaria is prevalent. Since both the function and interaction of many genes are still not fully understood then it would be unwise to remove them permanently as they may prove to be beneficial later (Saiki *et al.,* 1988).

Experiments using germline gene therapy in animals have been underway for a few years. In the initial demonstration of the use of the technique the human genes for growth hormone along with a regulator were inserted into mice embryos with the resultant expression in the recipient mice being a doubling of their body size. However the technology still has a high failure rate in terms of the large losses of egg cells, and the failure to achieve any expression (Vijayanathan *et al.,* 2002). With the way genetic information is transmitted in humans the genetic disease may not be expressed in the children, as they will only receive one of their pair of genes from the affected parent and would only be a carrier of the disease (Veiseh *et al.,* 2009). Mice that were lacking the gene for the synthesis of gonadotrophic releasing hormone were used in these trials, in which DNA containing the correct gene was injected into fertilized eggs. The successfully treated animals became normal for the synthesis of gonadotropic releasing hormone. Although germ-line therapy is not yet safe enough to use on humans, we must consider technology before it is ready to be used especially when such high stakes are involved. Germ line therapy will likely become available for human applications in the next 10 to 20 years according to scientists though it will likely appear under less threatening names. So the question is really not if this technology will be used but when and in what ways (Brivanlou and Darnell, 2002).

Germ-line gene therapy is a new technology being developed by scientists in the forefront of their fields. The final form that this technology will take depends on new advances and political influences by decision-makers. Concerns about gene therapy include safety, effectiveness, impact on the genome, funding of research, the future applications and direction of research (Cui *et al.,* 2012). The total elimination of all disease-causing alleles is an unrealistic goal, and is unobtainable. It is not possible to eradicate genetic disease completely as many people are carriers for genetic disease and are not aware of it, and many occur spontaneously as quickly as we would be able to get rid of them new mutations would occur. Germline therapy hold great potential for the treatment of many devastating genetic disorders (Kato *et al.,* 2005).

Given the inherent hazard it poses, germ-line therapy is a technology best postponed until adequate knowledge of the human genome is available, and implications of this process for the future of the human race. Then and only then can mankind safely decide whether germ-line engineering is the true answer to our genetic problems (Kamau *et al.,* 2006).

2. Somatic Gene Therapy: Somatic gene therapy is the transfer of genes into the somatic cells of the patient, such as cells of the bone marrow, and hence the new DNA does not enter the eggs or sperm. The genes transferred are usually normal alleles that could 'correct' the mutant or disease alleles of the recipient.

The technique of somatic gene therapy involves inserting a normal gene into the appropriate cells of an individual affected with a genetic disease, thereby permanently correcting the disorder (Neuman *et al.,* 1982). The targeted cells may be bone marrow cells, which are easily isolated and re-implanted. Bone marrow cells continue to divide for a person's whole life to produce blood cells, so this approach is useful only if the gene you want to deliver has a biological role in the blood. Delivery of a gene that has a biological role in the lungs, muscle, or liver would have to occur within those targeted organs. In many cases,

accessing the appropriate tissue or, if the gene is required in multiple tissues (e.g. muscles throughout the body) ensuring it can be delivered where it is needed, is a major problem (Sakaguchi *et al.,* 2008).

Somatic cells are non-reproductive. Somatic cell therapy is viewed as a more conservative, safer approach because it affects only the targeted cells in the patient, and is not passed on to future generations. In other words, the therapeutic effect of gene therapy ends with the individual who receives the therapy. However, this type of therapy presents unique problems of its own. Often the effects of somatic cell therapy are short-lived. From the fact that living cells of most tissues ultimately die and are replaced by new cells, repeated treatments over the course of the individual's life span are required to maintain the therapeutic effect (Pathak *et al*., 2009). Transporting the gene to the target cells or tissue is also problematic. Regardless of these difficulties, somatic cell gene therapy is appropriate and acceptable for many disorders, including cystic fibrosis, muscular dystrophy, cancer, and certain infectious diseases. Clinicians can even perform this therapy in vitro, potentially correcting or treating a life-threatening disorder that may significantly impair a baby's health or development if not treated before birth. Somatic gene therapy is restricted to the actual patient and is not passed on to his or her children (Cole-Strauss *et al.,* 1999). All gene therapy to date on humans has been directed at somatic cells, whereas germ-line engineering in humans remains controversial and prohibited in for instance the European Union. Somatic gene therapy can be split into two broad categories:

a) Ex vivo: which means exterior (where cells are modified outside the body and then transplanted back in again). In some gene therapy clinical trials, cells from the patient's blood or bone marrow are removed and grown in the laboratory. Cells are exposed to the virus that is carrying the desired gene. The virus enters into the cells and inserts the desired gene into the cells' DNA. The cells grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene therapy is called *ex vivo* (Figure 3), because the cells are treated outside the organism (Cheng *et al.,* 1994).

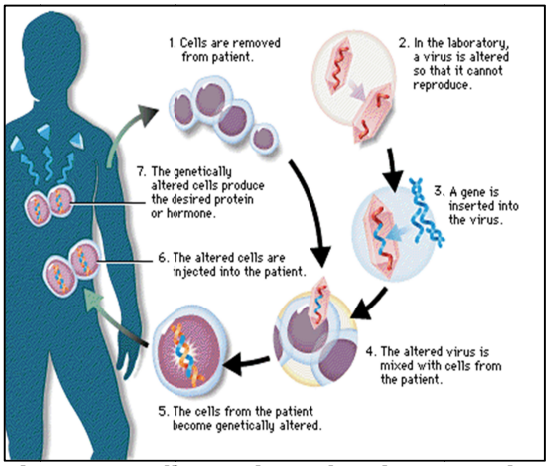

Figure 3: Illustration showing ex vivo form of somatic gene therapy. Source: ONGSIMEI (2016)

b) In vivo: which means interior (where genes are changed in cells still in the body). This form of gene therapy is called in vivo (Figure 4), because the gene is transferred to cells inside the patient's body (Cole-Strauss *et al.,* 1999).

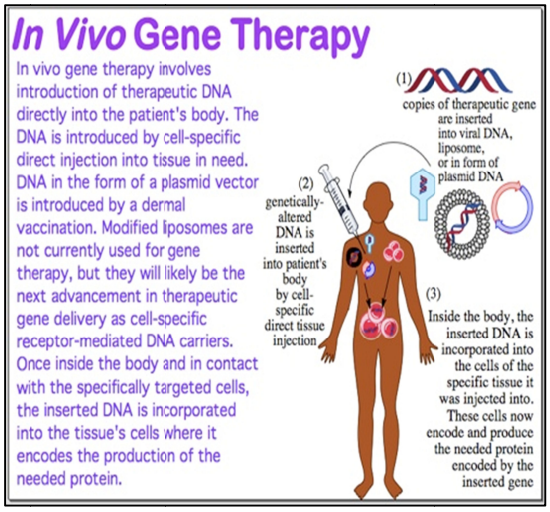

Figure 4: Illustration showing in vivo form of somatic gene therapy. Source: GURMUNSINGH (2018)

Viral vectors that are used in somatic gene therapy experiments include: retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses.

i) Retroviruses: Retroviruses were the first viruses used as vectors in gene therapy experiments. They are unusual because instead of using DNA to carry their genetic information to the cell's protein-making machinery, retroviruses use a related material called ribonucleic acid (RNA) as their primary carrier of genetic information. When retroviruses invade a cell, they use an enzyme called reverse transcriptase to make a DNA copy of their genes. Other enzymes then incorporate this DNA copy into the infected cell's DNA (Cheng *et al.,* 1994). Although retroviruses have been used in most gene therapy experiments so far, they still present many problems. Retroviruses can invade only cells that are actively dividing, limiting potential targets for therapy to blood cells, skin cells, stem cells, and other fast-growing tissues. In addition, the viruses have no specific targets in the infected cells' chromosomes. As a result, the genes they carry are inserted in a haphazard manner (Caplen *et al.,* 1995). Ideally, retroviruses insert genes into the middle of a strand of DNA that does not contain other genes. The genes might, however, be inserted smack in the middle of a crucial gene, rendering it defective and blocking key cellular functions, causing more damage than repair. Retroviruses could also integrate new genes into a stretch of DNA where they could cause cancer. Despite the presence of promoters, moreover, the added genes typically do not produce sufficient amounts of proteins to effectively treat disease. In addition, the patient's body generally recognizes retroviruses as foreign invaders, provoking adverse immune responses (Djikmans *et al.,* 2004).

As researchers have grown more confident, however, they have begun injecting altered retroviruses directly into tissues where the corrected genes are needed and have, so far, observed few problems. In clinical trials of patients with cystic fibrosis, a disease in which a mutated gene impairs lung function, healthy genes are inserted directly into the lining of bronchial tubes (Kato *et al*., 2005). In studies of animals, researchers have used retroviruses to inject genes directly into muscle tissue to learn if the genes will produce normal muscle proteins. Researchers hope this treatment will one day help people with muscular dystrophy (Lehrman, 1999).

ii) Adenoviruses: To avoid the problem of inserting genes at the wrong sites, some researchers have turned to other types of viruses, such as the adenoviruses, which cause the common cold. Stripped of their disease-causing genes, adenoviruses take healthy genes into the nucleus of cells, where the DNA is located, but do not usually integrate them into a cell's DNA. Researchers thus trade safety for impermanence, because the genes persist in the cell's DNA only for days to weeks (Mayeux, 2005). Adenoviruses can also infect a broader variety of cells than retroviruses do, including cells that divide more slowly, such as lung cells. However, adenoviruses are also more likely to be attacked by the patient's immune system, and the high levels of virus required for treatment often provoke an undesirable inflammatory response. Despite these drawbacks, adenoviruses have been used in attempts to treat cancers of the liver and ovaries (Kato *et al*., 2005).

iii) Adeno associated virus: One of the most promising potential gene-delivery systems, or vectors, is a recently discovered virus called the adeno-associated virus, which infects a broad range of cells, including both dividing and non-dividing cells. Researchers believe that most humans carry adeno-associated viruses, which do not cause disease and do not provoke an immune response. Scientists have demonstrated that the adeno-associated virus can be used to correct genetic defects in animals. It is now being used in preliminary studies to treat hemophilia, a hereditary blood disease, in humans (Robrish, 1999). The chief drawback of the adeno-associated virus is that it is small, carrying only two genes in its natural state. Its payload is therefore relatively limited. It can produce unintended genetic damage because the adeno-associated virus inserts its genes directly into the host cell's DNA. Researchers have also had difficulties manufacturing large quantities of the altered virus.

iv) Herpes simplex virus: Scientists have found that the herpes simplex virus, the cause of the common cold sore, has a very large genome compared to other virus vectors. This large genome enables scientists to insert more than one therapeutic gene into a single virus, paving the way for the treatment of disorders caused by more than one gene defect. The virus makes an ideal vector because it can infect a wide variety of tissues, including muscle, tumor, liver, pancreas, nerve, and lung cells (Sails, 2004). One problem with using herpes simplex virus is that the virus is cytopathic—that is, it kills the cells that it infects. In addition, the virus can cause encephalitis (inflammation of the brain) if it replicates freely in the brain. Scientists are developing a form of herpes simplex virus in which the genes that direct the virus's replication and cell-killing abilities have been removed (Saiki *et al*., 1985).

3. Chimeraplasty: Chimeraplasty, also known as targeted gene correction, is a technique in which a synthetically created molecule consisting of both RNA and DNA is used to repair single base pair mutations, deletions, or insertions in DNA (Alberts *et al*., 2002). The technique of chimeraplasty was developed in the lab of Drs. Eric B. Kmiec, Kyonggeun Yoon and Allyson Cole-Strauss at Thomas Jefferson University in Philadelphia, Pennsylvania. Kmiec studies homologous recombination and he realized that the rate of recombination is increased for active genes being transcribed into mRNAs. This led him to question whether synthetic RNA could be used in gene repair by tricking the cell to incorporate good DNA into mutated sites (Cole-Strauss *et al*., 1999).

A chimeraplast consists of a paper-clip shaped, double-stranded stretch of DNA interspersed with short strands of RNA. The design of the chimeraplast is a result of the discovery that hybrids of RNA and DNA are very active in homologous pairing reactions (Yoon *et al*., 1996). Furthermore, it was found that the hairpin caps at the ends of the molecules do not impede base pairing with target genes. The purpose of the short RNA strands is to activate the oligonucleotide ("oligo") for recombination, and the hairpin caps protect the molecule from destabilization or destruction by exonucleases or cellular helicases (Stephenson, 1999). Finally, the ribose of the RNA is 2'-O-methyl modified to add protection against cleavage by RNase H activities (Cole-Strauss *et al*., 1996).

When designing a chimeric oligonucleotide, there is a replication of a short portion of the base sequence of the target gene surrounding the base pair mutation so that it aligns perfectly with the gene, except for the one base pair where the mutation occurs (Jaroslav *et al*., 2002). In this location, the correct base is substituted into the oligonucleotide. Therefore, when the chimera inserts between the strands of target DNA, the mismatched base pair is recognized by the endogenous repair system, and the sequence is changed in either the chimeraplast (using the target DNA as a template), or in the target DNA (using the chimera as a template) (Robrish, 1999).In its mechanism of repair, chimeric oligonucleotides probably work by pairing with a plasmid target based on homology using DNA pairing enzymes and complexes. After pairing, endogenous repair machinery recognizes the mismatch between the gene and the chimera, and uses mismatch repair to correct the "spelling mistake" by using the chimera as the template sequence. Then, the chimera decays, leaving only the corrected target DNA (Smaglik, 2006).

For *in vivo* repair, the oligonucleotides can be attached to organ-specific ligands. Liposomes and synthetic polymers are also used to deliver chimeric molecules to the appropriate cells or tissue. In plants, microscopic gold particles are coated with the chimeric molecules and fired into cells (Sails, 2004). In all cases, the oligonucleotides that enter the nucleus can repair point mutations within the cell after pairing with their sequence-specific target DNA by causing the cell's repair machinery to perform mismatch repair on the point mutation. After correction, the chimera decays, leaving the corrected gene (Smaglik, 2006).

Techniques/Protocols in Gene Therapy

1. **Polymerase Chain Reaction:** Polymerase chain reaction (PCR) is a common and often indispensible technique used in medical and

biological research laboratories for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases (Lawyer *et al*., 1993). The method/protocol relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification (Park, 2004). As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations (Pierce and Wangh, 2007).

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. The DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis (Sarkar *et al*., 1990). Majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions (Arena *et al*., 2011). PCR is used to amplify specific region of DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. The reaction produces limited amount of final amplified product that is governed by the available reagents in the reaction and the feedback-inhibition of the reaction products (Chien *et al*., 1976).

A basic PCR set up requires several components and reagents. These components are: (i) DNA template that contains the DNA region (target) to be amplified, (ii) two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target, (iii) taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C, (iv) deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building blocks through which the DNA polymerase synthesizes a new DNA strand, (v) buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, (vi) divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis and (vii) monovalent cation potassium ions (Innis *et al.,* 1988; Isenbarger *et al.,* 2008).

The PCR is commonly carried out in a reaction volume of 10 – 200 µl in small reaction tubes (0.2 – 0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the required temperatures at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes are favorable thermal conductors that allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube (Lawyer *et al*., 1993).

Typically, PCR consists of a series of 20 – 40 repeated temperature changes, called cycles, with each cycle commonly consisting of

2 – 3 discrete temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (about 90°C), and followed by one hold at the end for final product extension or brief storage (Park, 2004). The temperatures used and the length of time they are applied in each cycle depend on a number of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

Initialization step: This step consists of heating the reaction to a temperature of between 94 – 96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1 – 9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Isenbarger *et al*., 2008).

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to temperature between 94 – 98 °C for 20 – 30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules (Lawyer *et al*., 1993; Isenbarger *et al.,* 2008).

Annealing step: The reaction temperature is lowered to 50 – 65 °C for 20 – 40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3 - 5 ^{o}C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation (Pierce and Wangh, 2007).

Extension/elongation step: The temperature at this step is determined by the type of DNA polymerase used; Taq polymerase has its optimum activity temperature at 75 – 80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. Usually, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation: This step is occasionally performed at a temperature between 70 – 74 °C for 5 – 15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction (Park, 2004).

To check whether the PCR generated the anticipated DNA fragment (sometimes referred to as the amplimer or amplicon). Agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. The PCR process can be divided into three stages:

Exponential amplification: At every cycle, the quantity of product is doubled (assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA need to be present.

Leveling off stage: The reaction slows down as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

Plateau: No more product accumulates due to exhaustion of reagents and enzyme (Pierce and Wangh, 2007; Isenbarger *et al*., 2008).

PCR Optimization

In practice, PCR can fail due to various reasons. In most cases due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with laboratory protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants (Chien *et al*., 1976). This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plastic ware, and thoroughly cleaning the work surface between reaction setups (Rychlik *et al*., 1990). Primer design techniques are important in improving PCR product yield and in avoiding the formation of spurious DNA products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA (Park, 2004). Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design (Pavlov *et al.,* 2004; Isenbarger *et al.,* 2008).

Applications of PCR

a. Selective DNA isolation: PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes and DNA cloning, which require larger amounts of DNA, representing a specific DNA region (Park, 2004). PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting materials for gene therapy protocols.

Other applications of PCR include (i) DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, and (ii) isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism (Sanger *et al*., 1977). *Escherichia coli* colonies can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism (forensic entomology) by comparing experimental DNAs through different PCR-based methods (Cheng *et al*., 1994).

b. Amplification and quantification of DNA: Because the regions of DNA that it targeted are amplified by PCR; PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old (Mullis, 1990; Park, 2004). Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification (Pavlov *et al*., 2006).

c. PCR in diagnosis of diseases: PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000-fold higher than that of other methods (Pavlov *et al*., 2004).

PCR aids the identification of non-cultivatable or slow-growing microorganisms such as anaerobic bacteria, mycobacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes (Pavlov *et al*., 2006; Pierce and Wangh, 2007).

Viral DNA can likewise be detected by PCR. The primers used must be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in treatment. The quantity or amount of virus ("viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques (Sails, 2004).

Variations of the PCR Technique in Gene Therapy

Allele-specific PCR: a diagnostic or cloning technique based on single-nucleotide polymorphisms (SNP) (single-base differences in DNA). SNP requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence (Lawyer *et al*., 1993).

Asymmetric PCR: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out normally, but with excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification of this process, called Linear-After-The-Exponential-PCR (LATE-PCR) uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction (Pierce and Wangh, 2007).

Multiplex PCR: is the use of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more *time* to perform. Annealing temperatures for each of the primer sets must be fully optimized to work correctly within a single reaction and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis (Pierce and Wangh, 2007; Isenbarger *et al.,* 2008).

Nested PCR: increases the specificity of DNA amplification, by reducing background arising from non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then use in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences (Sarker *et al.,* 1990; Pierce and Wangh, 2007).

Nanoparticles

Transgenic technology has been widely used in breeding new plant and animal varieties. Effective gene delivery into the target cells is an essential step in these practices (Jaroslav *et al*., 2002). Both viral and nonviral vectors have been used for gene delivery especially with regards to gene therapy. Nanoparticles as gene vectors, due to their reduced immunogenicity, improved safety and the ability to carry larger DNA loads has become an attractive alternative in gene delivery. Cationic liposomes have been widely used as non-viral gene vectors, but, because of its cell-specific, easily inactivation of serum protein, and other reasons, its scope of application is also limited (Mehrdad *et al*., 2008).

Because of their unique physical and chemical properties, nanoparticles have been widely used in the field of drug and gene delivery to target cells. As a novel kind of gene vectors, nanoparticles have many advantages (Pathak *et al*., 2009). DNA molecules can be protected by nanoparticles against nuclease degradation by wrapping and condensing nucleic acids, realize the specificity of gene delivery by conjugating with special target molecules, and achieve controlled release of DNA effectively extending the duration of action (Veiseh *et al*., 2009). Besides the advantages of ordinary nanoparticles, other nanoparticles like magnetic nanoparticles are superparamagnetic. They can induce the DNA moving and condensing to the target cells in the presence of a magnetic field, which can significantly improve their efficiency as gene carriers (Vijayanathan *et al*., 2002).

Although the application of magnetic nanoparticles in gene diagnosis and therapy has been studied extensively, the gene transfer systems based on magnetic nanoparticles for gene delivery into mammalian cells are few studied (Plank *et al*., 2003a). Effective gene delivery into mammalian cells is an essential step in reproductive cloning using somatic cell nuclear transfer technique. New advances in this field report a new approach to deliver genes to porcine somatic cells using PEI-modified magnetic nanoparticles as gene vector (Plank *et al*., 2003b). To access the potential of magnetic nanoparticles as gene transfer vectors in transgenic animal production, PEI-modified Fe_3O_4 magnetic nanoparticles were employed to transfer reporter gene into somatic cells for gene delivery efficiency (Yang *et al*., 2008; Cui *et al.,* 2012).

The PEI-modified magnetic nanoparticles are spherical in shape with an average diameter of about 150 nm. The surface of the nanoparticle becomes coarse and rough, and the average diameter increases to 200 nm after conjugated with the plasmid DNA. The zeta potential of nanoparticle/DNA complexes drops down from +29.4 mV to +23.1 mV after the conjugation. Agarose gel electrophoresis experiments show that DNA plasmids can be loaded and protected effectively against degradation of exonuclease and endonuclease by PEI-modified magnetic nanoparticles (Kamau *et al*., 2006). It is found that the efficiency of gene delivery to the target sites is/are not only affected by the mass ratio of nanoparticle/DNA, but also the amount of the nanoparticle/DNA complexes. Based on the analysis of the PEI-modified magnetic nanoparticles, the combination of nanoparticles has a good ability to bind with plasmid DNA (Kumari *et al*., 2010). Moreover, the formation of nanoparticle/DNA complexes can protect DNA against enzyme degradation. Such advantages of the PEI-modified magnetic nanoparticles make it have a potential strength as vector for gene delivery (Kato *et al.,* 2005; Cui *et al*., 2012).

The nanoparticles are used to deliver green fluorescent protein expression carrier (pEGFP-N1) into PK-15 cells as gene vectors. The expression of green fluorescent protein gene (GFP) is detected by fluorescence microscope. The efficiency of GFP expression can be enhanced significantly in the presence of a magnetic field (Kaneda, 2003). The reason is that the plasmid DNA can be directionally delivered to the receptor cells by magnetic nanoparticle under the magnetic field, which condensed the concentration of nanoparticle/DNA complexes on the surface of the cells, resulting in the increased cellular endocytosis of the complexes (Cui *et al*., 2012).

The delivery of genes of interest into PK-15 and PEF cells using magnetic nanoparticles provides an important experimental basis for the application of the magnetic nanoparticles as gene transfer vector for somatic cells, thereby providing the foundation study for its application in gene therapy as well as an important technique in breeding new transgenic cloned plants and animals (Scherer *et al*., 2002; Kaneda, 2003).

Sonoporation

Sonoporation, or cellular sonication, is the use of sound (typically ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. This technique is usually used in molecular biology and non-viral gene therapy in order to allow uptake of large molecules such as DNA into the cell, in a cell disruption process

called cell transfection or cell transformation (Arena *et al*., 2011). Sonoporation employs the acoustic cavitation of microbubbles to enhance delivery of these large molecules (Figure 5).

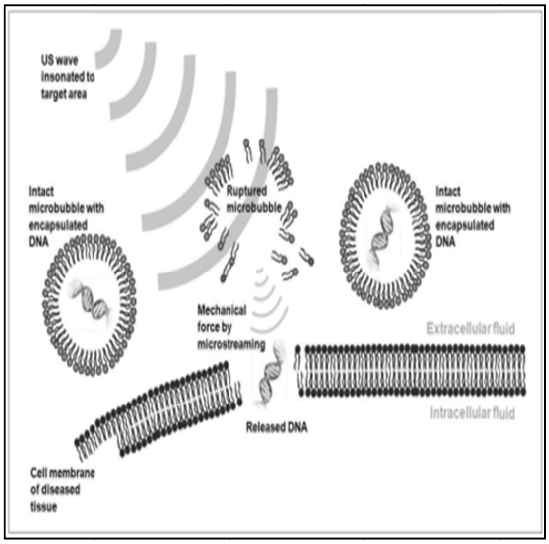

Figure 5: Schematic illustration showing sonoporation. Source: Kim *et al.* (2008)

The bioactivity of sonoporation is similar to, and in some cases found superior to, electroporation. Extended exposure to low-frequency (<MHz) ultrasound has been demonstrated to result in complete cellular death (rupturing), thus cellular viability must also be accounted for when employing this technique (Church, 2005).

Sonoporation is under active study for the introduction of foreign genes in tissue culture cells, especially mammalian cells. Sonoporation is also being studied for use in targeted gene therapy *in vivo*, in a medical treatment scenario whereby a patient is given modified DNA, and an ultrasonic transducer might target this modified DNA into specific regions of the patient's body (Song *et al*., 2007).

Sonoporation is performed with a dedicated sonoporator. Sonoporation may also be performed with custom-built piezoelectric transducers connected to bench-top function generators and acoustic amplifiers. Standard ultrasound medical devices may also be used in some applications (Church, 2005).

Measurement of the acoustics used in sonoporation is listed in terms of mechanical index, which quantifies the likelihood that exposure to diagnostic ultrasound will produce an adverse biological effect by a non-thermal action based on pressure. Sonoporation uses microbubbles for significantly enhancing cell transfection, and in some cases is required for DNA (Jaroslav *et al*., 2002).

Electroporation

Electroporation, or electropermeabilization, is a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field (Becker and Kuznetov, 2007). It is usually used in molecular biology as a way of introducing some substance into a cell, such as loading it with a molecular probe, a drug that can change the cell's function, or a piece of coding DNA (Djikmans *et al*., 2004).

Electroporation is a dynamic phenomenon that depends on the local transmembrane voltage at each point on the cell membrane. It is generally accepted that for a given pulse duration and shape, a specific transmembrane voltage threshold exists for the manifestation of the electroporation phenomenon (from 0.5 V to 1 V) (Miklavcic *et al.,* 2010). This leads to the definition of an electric field magnitude threshold for electroporation (E_{th}). That is, only the cells within areas where $E \geqq E_{th}$ are electroporated. If a second threshold (E_{ir}) is reached or surpassed, electroporation will compromise the viability of the cells, i.e., irreversible electroporation (Arena *et al*., 2011).

In molecular biology, the process of electroporation is often used for the *transformation* of bacteria, yeast, and plant protoplasts. In addition to the lipid membranes, bacteria also have cell walls which are different from the lipid membranes and are made of peptidoglycan and its derivatives (Pathak *et al*., 2009). However, the walls are naturally porous and only act as stiff shells that protect bacteria from severe environmental impacts. If bacteria and plasmids are mixed together, the plasmids can be transferred into the cell after

electroporation. Several hundred volts across a distance of several millimeters are typically used in this process. Afterwards, the cells have to be handled carefully until they have had a chance to divide producing new cells that contain reproduced plasmids. This process is approximately ten times as effective as chemical transformation (Neumann *et al.,* 1982; Sugar and Neumann, 1984).

This procedure is also highly efficient for the introduction of foreign genes in tissue culture cells, especially mammalian cells. For example, it is used in the process of producing knockout mice, as well as in tumor treatment, gene therapy, and cell-based therapy. The process of introducing foreign DNAs into eukaryotic cells is known as transfection. Electroporation is done with electroporators, appliances that create an electro-magnetic field in the cell solution. The cell suspension is pipetted into a glass or plastic cuvette which has two aluminum electrodes on its sides (Garcia *et al*., 2010).

For bacterial electroporation, typically a suspension of around 50 microliters is used. Prior to electroporation it is mixed with the plasmid to be transformed. The mixture is pipetted into the cuvette, the voltage and capacitance are set, and the cuvette is inserted into the electroporator. Immediately after electroporation, one milliliter of liquid medium is added to the bacteria (in the cuvette or in an eppendorf tube), and the tube is incubated at the bacteria's optimal temperature for an hour or more to allow recovery of the cells and expression of antibiotic resistance, followed by spreading on agar plates (Garcia *et al*., 2011). The success of the electroporation depends greatly on the purity of the plasmid solution, most especially on its salt content. Solutions with high salt concentrations might cause an electrical discharge (known as arcing), which often reduces the viability of the bacteria (Thomson *et al*., 2011).

For further detailed investigation of the process, more attention should be paid to the output impedance of the porator device and the input impedance of the cells suspension (e.g. salt content). As the process needs direct electrical contact between the electrodes and the suspension, and is inoperable with isolated electrodes, obviously the process involves certain electrolytic effects, due to small currents and not only fields (Garcia *et al*., 2010a).

Gene Gun

A gene gun or a biolistic particle delivery system, originally designed for plant transformation, a device for injecting cells with genetic information. The payload is an elemental particle of a heavy metal coated with plasmid DNA. This technique is often simply referred to as bioballistics or biolistics. The gene gun is able to transform almost any type of cell, including plants, and is not limited to genetic material of the nucleus: it can also transform organelles, including plastids (Wolf *et al*., 2002). It was invented by John C Sanford, Ed Wolf and Nelson Allen at Cornell, and Ted Klein of DuPont, between 1983 and 1986. The original target was onions (chosen because of their large cell size) and it was used to deliver particles coated with a marker gene. Genetic transformation was then proven when the onion tissue expressed the gene. Gene guns are so far mostly applied to plant cells. However, there is much potential use in animals and humans as well. Gene guns have also been used to deliver DNA vaccines (Yao *et al*., 2006). The delivery of plasmids into rat neurons through the use of a gene gun, specifically DRG neurons, is also used as a pharmacological precursor in studying the effects of neurodegenerative diseases such as Alzheimer's disease (Gan, 1989).

Plant transformation using particle bombardment/gene gun follows the same outline as *Agrobacterium*-mediated method (Figure 6). The steps taken include: i) isolate the genes of interest from the source organism; ii) develop a functional transgenic construct including the gene of interest; promoters to drive expression; codon modification, if needed to increase successful production of protein; and marker genes to facilitate tracking of the introduced genes in the host plant; iii) incorporate into a useful plasmid; iv) introduce the transgenes into plant cells; v) regenerate the plants cells; and vi) test trait performance or

gene expression at lab, greenhouse and field level (Gan, 1989).

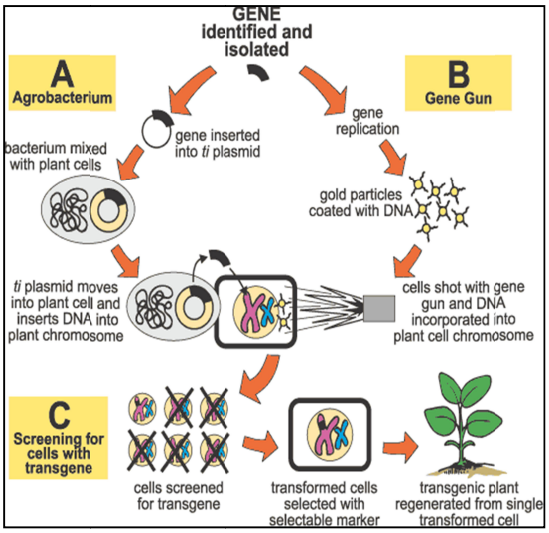

Figure 6: Schematic illustration of biolistic particle delivery system in plants. Source: Hingra (2018)

The particle bombardment method begins with coating tungsten or gold particles (micro-projectiles) with plasmid DNA. The coated particles are coated on a macro-projectile, which is accelerated with air pressure and shot into plant tissue on a petri plate. A perforated plate is used to stop the macro-projectile, while allowing the micro-projectiles to pass through to the cells on the other side. As the micro-projectiles enter the cells, the transgenes are released from the particle surface and may incorporate into the chromosomal DNA of the cells. Selectable markers are used to identify the cells that take-up the transgene. The transformed plant cells are then regenerated into whole plants using tissue culture techniques (Yao *et al*., 2006). Particle bombardment also plays an important role in the transformation of organelles such as chloroplasts, which enables engineering of organelle-encoded herbicide or pesticide resistances in crop plants and to study photosynthetic processes (Wolf *et al*., 2002). Limitations to the particle bombardment method with regards to Agrobacterium-mediated transformation include (i) frequent integration of multiple copies of the transgene at a single insertion site, (ii) rearrangement of the inserted genes, and (iii) incorporation of the transgene at multiple insertion sites. These multiple copies can be linked to silencing of the transgene in subsequent progeny (Gan, 1989; Yao *et al*., 2006).

Applications of Gene Therapy

Genetic engineering is an indispensible tool for natural scientists. Genes and other genetic information from a wide range of organisms are transformed into bacteria for storage and modification, creating genetically modified bacteria in the process. Bacteria are cheap, easy to grow, multiply quickly, relatively easy to transform and can be stored at -80°C almost indefinitely. Once a gene is isolated it can be stored inside the bacteria providing an unlimited supply for research (Lodish *et al*., 2000).

Gene therapy finds applications in medicine, agriculture and in the production of genetically engineered organisms in order to discover the functions of certain genes (Mayeux, 2005). This could be the effect on the phenotype of the organism, where the gene is expressed or what other genes it interacts with.

1. Physiological applications: One promising application of gene therapy is in treating type I diabetes. Current research used an adenovirus as a vector to deliver the gene for hepatocyte growth factor (HGF) to pancreatic islet cells removed from rats. They injected the altered cells into diabetic rats and, within a day, the rats were controlling their blood glucose levels better than the control rats. This model mimics the transplantation of islet cells in humans and shows that the addition of the HGF gene greatly enhances the islet cells' function and survival.

In May 2006, a team of scientists reported a breakthrough for gene therapy, in which they developed a way to prevent the immune system from rejecting a newly delivered gene. Delivery of 'normal' gene has been difficult because the immune system recognizes the new gene as foreign and rejects the cells carrying it. This problem was overcome utilizing a newly uncovered network of genes regulated by molecules known as microRNAs. The use of this natural function of microRNA is to selectively turn off the identity of the

therapeutic gene in cells of the immune system and prevent the gene from being found and destroyed. The output of the study has important implications for the treatment of hemophilia and other genetic diseases by gene therapy.

RNA interference or gene silencing may be a new way to treat Huntington's disease. Short pieces of double-stranded RNA and/or short interfering RNAs (siRNAs) are used by cells to degrade RNA of a particular sequence. If a siRNA is designed to match the RNA copied from a faulty gene, then the abnormal protein product of that gene will not be produced.

Gene therapy have been successfully used to treat metastatic melanoma using killer T-cells genetically retargeted to attack the cancer cells. This study constitutes one of the first demonstrations that gene therapy can and will be effective in treating cancer. Gene therapy has been used to treat Leber's Congenital Amaurosis (LCA), a rare inherited retinal degenerative disorder that causes blindness in children. The patients had a defect in the RPE65 gene, which was replaced with a functional copy using adeno-associated virus. In all the clinical trials, patients recovered functional vision without apparent side-effects. These studies, which used adeno-associated virus, have spawned a number of new studies investigating gene therapy for human retinal disease.

2. Agricultural applications: Gene therapy has seen basic applications in the field of agriculture and most especially in animal health and production. This has helped to enhance the genetic capabilities of farm animals. Applications of gene therapy in animal production have led to the creation of farm animals with rapid growth and maturity rates, increased meat/beef production and increased immunity of farm animals with respect to common diseases which these animals are normally exposed to etc.

3. Production of genetically modified organisms (GMOs): GMOs are organisms whose genetic materials have been altered as a result of the incorporation of DNA molecules from different sources, leading to the creation of organisms with novel genes. GMOs are useful in biological and biomedical research studies. Gene therapy focuses its application on GMOs by trying to find out more about these organisms using the following means:

i. Loss of function experiments: This occurs in gene knockout experiment, in which an organism is engineered to lack the activity of one or more genes. A knockout experiment involves the creation and manipulation of a DNA construct *in vitro*, which, in a simple knockout, consists of a copy of the desired gene, which has been altered such that it is non-functional. Embryonic stem cells incorporate the altered gene, which replaces the already present functional copy. These stem cells are injected into blastocysts, which are implanted into surrogate mothers. This allows the experimenter to analyze the defects caused by this mutation and thereby determine the role of particular genes. It is used especially frequently in developmental biology. Another method useful in organisms such as *Drosophila*, is to induce mutations in a large population and then screen the progeny for the desired mutation. A similar process can be used in both plants and prokaryotes (Pavlov *et al*., 2004).

ii. Gain of function experiments: This is the logical counterpart of knockouts. Gains of function experiments are sometimes performed in conjunction with knockout experiments to more finely establish the function of the desired gene. The process is much the same as that in knockout engineering, except that the construct is designed to increase the function of the gene, usually by providing extra copies of the gene or inducing synthesis of the protein more frequently (Thomson *et al*., 2011).

iii. **Tracking experiments:** This seeks to gain information about the localization and interaction of the desired protein. Tracking experiment is done by replacing the wild-type gene with a 'fusion' gene, which is a juxtaposition of the wild-type gene with a reporting element such as green fluorescent protein (GFP) that will allow easy visualization of the products of the genetic modification (Alberts *et al*., 2002). While this is a useful technique,

the manipulation can destroy the function of the gene, thus creating secondary effects and possibly calling into question the outcome of the experiment. More sophisticated techniques are now in development that can track protein products without mitigating their function, such as the addition of small sequences that will serve as binding motifs to enhance the activities of monoclonal antibodies.

iv. Expression studies: This aims to discover where and when specific proteins are produced. In these experiments, the DNA sequence before the DNA that codes for a protein, known as a gene's promoter, is reintroduced into an organism with the protein coding region replaced by a reporter gene such as GFP or an enzyme that catalyzes the production of a dye (Caplen *et al*., 1995). Thus the time and place where a particular protein is produced can be observed. Expression studies can be taken a step further by changing the promoter to find which pieces are crucial for the proper expression of the gene and are actually bound by transcription factor proteins; this process is known as promoter bashing (Dijkmans *et al*., 2004).

A technique called the non-thermal irreversible electroporation (N-TIRE) has been successfully used in treating many different types of tumors and other unwanted tissue. This procedure is done using small electrodes (about 1mm in diameter), placed either inside or surrounding the target tissue to apply short, repetitive bursts of electricity at a predetermined voltage and frequency (Kaneda, 2003). The bursts of electricity increases the resting transmembrane potential (TMP), so that nanopores are formed in the plasma membrane. When the electricity applied to the tissue is above the electric field threshold of the target tissue, the cells become permanently permeable from the formation of nanopores. As a result, the cells are unable to repair the damage and die due to a loss of homeostasis. N-TIRE is unique to other tumor ablation techniques in that it does not create thermal damage to the tissues around it (Pathak *et al*., 2009; Miklavcic *et al*., 2010).

One major advantage of using N-TIRE is that, when done correctly according to careful calculations and protocols, it only affects the target tissue. Proteins, the extracellular matrix, and critical structures such as blood vessels and nerves are all unaffected and left healthy by this treatment. This allows for quicker recovery and facilitates rapid replacement of dead tumor cells with healthy cells (Garcia *et al.,* 2011).

Although medical scientists are not ready to routinely use irreversible electroporation to treat complicated tumors in humans, N-TIRE is more commonly used to treat simple cutaneous or subcutaneous tumors in humans. In addition to treating cutaneous and subcutaneous tumors, there have been attempts at using this technology to treat prostate, lung, kidney and liver cancer in humans. However, scientists are still in the process of understanding this technology and its effect on animals and humans (Garcia *et al*., 2010b). A recent study tested the safety of N-TIRE on humans suffering from lung, kidney or liver tumors. Of the 69 tumors treated, 49 of them achieved complete tumor ablation. The most successful treatment rate occurred in liver tumors. This study provides encouraging evidence for the future of the use of N-TIRE as a method of treating cancer in humans, hence becoming a vital tool in gene therapy (Pathak *et al*., 2009).

Physiologically, electroporation as a protocol in gene therapy can also be used to help deliver drugs or genes into the cell by applying short and intense electric pulses that transiently permeabilize cell membrane, thus allowing transport of molecules otherwise not transported through a cellular membrane. This procedure is referred to as electrochemotherapy when the molecule to be transported is a chemotherapeutic agent or gene electrotransfer when the molecule to be transported is DNA (Thomson *et al*., 2011).

Electroporation allows cellular introduction of large highly charged molecules such as DNA which would never passively diffuse across the hydrophobic bilayer core. This phenomenon indicates that the mechanism is the creation of nm-scale water-filled holes in the membrane (Vijayanathan *et al*., 2002). Although

electroporation and dielectric breakdown both result from application of an electric field, the mechanisms involved are fundamentally different. In dielectric breakdown the barrier material is ionized, creating a conductive pathway. The material alteration is thus chemical in nature. In contrast, during electroporation the lipid molecules are not chemically altered but simply shift position, opening up a pore which acts as the conductive pathway through the bilayer as it is filled with water (Miklavcic *et al*., 2010).

Problems and Prospects of Gene Therapy: Since the first clinical gene-therapy trial was conducted, much attention and considerable promise has been given to the field. There has been substantial public- and private-sector investment, as well as increasingly higher levels of research activity. Numerous preclinical animal-model studies have provided proofs of concept for multiple potential clinical applications. Also, major advances have been made in understanding vector biology and improving vector design and production with regards to gene therapy (Sakaguchi *et al*., 2008).

Problems Associated with Gene Therapy: Some of the problems associated with gene therapy include:

i. Short-lived nature of gene therapy: Before gene therapy can produce a permanent cure for any condition, the therapeutic DNA introduced into targeted cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. The problem 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.

ii. Immune response: Anytime a foreign object is introduced into human system, 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 much more difficult for gene therapy to be repeated in patients.

iii. 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.

iv. 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 those mentioned above would be especially difficult to effectively treat using gene therapy.

v. 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. One possible solution for this is to add a functional tumor suppressor gene onto the DNA to be integrated; however, this poses its own problems, since the longer the DNA is, the harder it is to integrate it efficiently into cell genomes (Lehrman, 1999; Pierce and Wangh, 2007; Khan *et al.,* 2008)

Prospects of Gene Therapy

There are a lot of prospects pertaining to gene therapy. Gene therapy as we all know it is not a 'cure all' option. It is not always successful but it means that a disease can be eradicated for a

person and their future offspring, so it is remedied in not just one generation but also in subsequent generations (Arena *et al*., 2011).

Gene therapy also has the potential to 'silence' a gene as in the case of a subject with HIV, which had not yet developed into AIDS, scientists could save them the pain and suffering of the disease by using gene therapy to 'silence' the disease before its onset. I believe that if the cynics and those who are skeptical to this technique were ever faced with cancer or a child born with a genetic disease, they would change their views. These skeptics would almost certainly choose gene therapy, especially if it was the last hope for them or one of their loved ones – as is the case for many gene therapy patients (Khan *et al*., 2008). In 2000, a team of medical doctors in Paris described results from a study involving two children suffering from a severe combined immunodeficiency disorder (SCID-XI), which had restricted them to life in an isolated environment (Kato *et al*., 2005). These investigators used a MoMLV vector to transfer a curative gene (γc cytokine receptor subunit) into the patients' lymphocytes ex vivo, and after amplification of the cells, returned them to the patients. Both patients were able to leave the hospital and resume normal lives (Yang, 2007).

Gene therapy has the potential to eliminate and prevent hereditary diseases such as cystic fibrosis and is a possible cure for heart disease, AIDS, Alziehmer's disease and cancer. This potential will go a long way to ensure better health for generations unborn, boost immune health in individuals with immune deficiency diseases, enhance agricultural productivity of farm animals, and in the production of genetically modified animals (GMOs) which will further help in medical and biomedical research.

Conclusion: Gene therapy is the use of DNA (as a pharmaceutical agent) to treat disease. It is also an experimental medical treatment that manipulates a gene or genes within cells in order to produce proteins that change the function of those cells. In gene therapy, DNA that encodes a therapeutic protein is packaged within a "vector" which is used to get the DNA inside the cells within the body. The acquired DNA becomes expressed by the cell machinery resulting in the production of a therapeutic protein which in turn treats the patient's diseases. The technology of gene therapy is based on the effective delivery of the corrective genes and to do this, scientists have developed gene delivery vehicles called vectors. The vectors encapsulate therapeutic genes for delivery into the target cells. Many of the vectors currently in use are based on attenuated or modified versions of viruses. Plasmids, which are circular pieces of DNA extracted from bacteria, are also used as vectors. The therapeutic gene to be transferred is extracted from the cell of a healthy individual. The gene is extracted by cutting the DNA using a restriction enzyme (restriction enzymes "digest" DNA at designated nucleotide locations along the DNA chain).There are a lot of techniques/protocols that are used in gene therapy. These techniques include the polymerase chain reaction (PCR), electroporation, nanoparticles, sonoporation etc. These protocols rely on different means to deliver the DNA of interest to the target sites within the genes. PCR utilizes heat-stable DNA polymerase such as Taq-polymerase, an enzyme that is originally isolated from the bacterium *Thermus aquaticus*. Nanoparticles as a gene vector have the ability to carry and transfect larger loads of DNA and this makes it attractive in gene delivery. Because of their unique physical and chemical properties, nanoparticles can protect DNA molecules against nuclease degradation. Sonoporation technique is a non-viral means of DNA delivery in the gene. This technique employs acoustic cavitation of microbubbles so as to enhance delivery of genes. Electroporation is a highly efficient technique for introduction of foreign genes in tissue culture in mammalian cells. The success of the electroporation depends greatly on the purity of the plasmid solution, especially on its salt content. Solutions with high salt concentrations might cause an electrical discharge (known as arcing), which often reduces the viability of the bacteria. Gene therapy finds applications in genetically engineered organisms in order to discover the functions of certain genes. This could be the

effect on the phenotype of the organism, where the gene is expressed or what other genes it interacts with. Gene therapy generally finds application in loss of function, gain of function, tracking and expression. Clinical trials of gene therapy experiments have seen advances in understanding vector biology and improving vector designs. Some problems associated with gene therapy include; short-lived nature of the therapy, problem of viral vectors, multigene disorders etc. Gene therapy experiments suffered a major setback as a result of intense criticisms and skepticisms following the death of an 18-year old man as result of massive immune reaction arising from the vector (Adenovirus, Ad5 vector) used in the clinical trial. The outcome of this trial led to a much tighter regulation of all trials relating to gene therapy. However there are other reports of successful clinical trials of gene therapy experiments involving children suffering from a severe combined immunodeficiency disorder (SCID-XI), which had restricted them to life in an isolated environment. These investigators used a MoMLV vector to transfer a curative gene (γc cytokine receptor subunit) into the patients' lymphocytes *ex vivo*, and after amplification of the cells, returned them to the patients. Both patients were able to leave the hospital and resume normal lives. The success of the SCID-XI trial likely reflects the path that gene therapy will follow during the next 1 to 2 decades: success, but with some complications. These experiences add further credence to the general viewpoint that gene therapy is a field in its infancy, and despite some pitfalls, it is well grounded in fundamental scientific principles with real clinical promise for the future.

REFERENCES

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. and WALTER, P. (2002). *Molecular Biology of the Cell.* 4th Edition, Garland Science, New York.

ARENA, C., SANO, M., ROSSMEISL, J., CALDWELL, J., GARCIA, P., RYLANDER, M. and DAVALOS, R. (2011). High-frequency irreversible electroporation (h-fire) for non-thermal ablation without muscle contraction. *Biomedical Engineering Online,* 10(102): 1 – 20.

AUSTRALIAN SCIENCE and MEDIA CENTRE (2010). DNA transfer prevents mitochondrial disease in humans. https://www.sciencemediacentre.co.nz/2010/04/15/dna-transfer-prevents-mitochondrial-disease-in-humans/ Accessed May 16, 2018.

BECKER, S. M. and KUZNETSOV, A. V. (2007). Local temperature rises influence *in vivo* electroporation pore development: a numerical stratum corneum lipid phase transition model. *Journal of Biomechanical Engineering,* 129(5): 712 – 717.

BERG, J. M., TYMOCZKO, J. L., STRYER, L. and CLARKE, N. D. (2002). DNA, RNA, and the Flow of Genetic Information: Amino Acids Are Encoded by Groups of Three Bases Starting from a Fixed Point. Chapter 5, *Biochemistry,* 5th Edition, W. H. Freeman and Company, New York.

BRIVANLOU, A. H. and DARNELL, J. E. (2002). Signal transduction and the control of gene expression. *Science,* 295(5556): 813 – 818.

CAPLEN, N. J., ALTON, E. W. F. W., MIDDLETON, P. G., SMITH, S. N., STEEL, D. M. and MUKONGE, F. M. (1995). Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Medicine*, 1(1): 39 – 46.

CHURCH, C. C. (2005). Frequency, pulse length, and the mechanical index. *Acoustics Research Letters Online,* 6(3): 162 – 168.

CHENG, S., FOCKLER, C., BARNES, W. M. and HIGUCHI, R. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proceedings of the National Academy of Sciences,* 91(12): 5695 – 5699.

CHIEN, A., EDGAR, D. B. and TRELA, J. M. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology,* 127(3): 1550 – 1557.

COGHLAN, A. (1999). Look, no new genes: A subtle technique tricks plant cells in

doing their own genetic engineering. *New Scientist,* 163(2197): 4.

COLE-STRAUSS, A., GAMPER, H., HOLLOMAN, W. K., MUNOZ, M., CHENG N. and KMIEC, E. B. (1999). Targeted gene repair directed by the chimeric RNA/DNA oligonucleotide in a mammalian cell-free extract. *Nucleic Acids Research,* 27(5): 1323 – 1330.

COLE-STRAUSS, A., YOON, K., XIANG, Y., BYRNE, B. C., RICE, M. C., GRYN, J., HOLLOMAN, W. K. and KMIEC, E. B. (1996). Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. *Science,* 273(5280): 1386 – 1389.

CUI, J., CUI, H., WANG, Y., SUN, C., LI, K., REN, H. and DU, W. (2012). Application of PEI-modified magnetic nanoparticles as gene transfer vector for the genetic modification of animals. *Advances in Materials Science and Engineering*, 2012: doi:10.1155/2012/764521

DIJKMANS, P. A., JUFFERMANS, L. J. M., MUSTERS, R. J. P., VAN WAMEL, A., TEN CATE, F. J., VAN GILST, W., VISSER, C. A., DE JONG, N. and KAMP, O. (2004). Microbubbles and ultrasound: from diagnosis to therapy. *European Journal of Echocardiography*, 5(4): 245 – 246.

FELGNER, P. L. and RINGOLD, G. M. (1989). Cationic liposome-mediated transfection. *Nature*, 337(6205): 387 – 388.

GAN, C. (1989). Gene gun accelerates DNA-coated particles to transform intact cells. *The Scientist,* 3(18): 25.

GARCIA, P., NEAL, R., ROSSMEISL, J. and DAVALOS, R. (2010). Non-thermal irreversible electroporation for deep intracranial disorders. *Paper presented at 32nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society,* Buenos Aires, Argentina.

GARCIA, P., ROSSMEISL, J. and DAVALOS, R. (2011). Electrical conductivity changes during irreversible electroporation treatment of brain cancer. *Paper presented at 33nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society,* Boston, Massachusetts.

GARCIA, P., ROSSMEISL, J., NEAL, R., ELLIS, T., OLSON, J., HENAO-GUERRERO, N., ROBERTSON, J. and DAVALOS, R. (2010b). Intracranial non-thermal irreversible electroporation in vivo analysis. *The Journal of Membrane Biology,* 236(1): 127 – 136.

GRIFFITHS, J. H., MILLER, J. H., SUZUKI, D. T., LEWONTIN, R. C. and GELBART, W. M. (2000). *An Introduction to Genetic Analysis.* 7th Edition, W. H. Freeman, New York.

GURA, T. (1999). Repairing the genome's spelling mistakes. *Science,* 285(5426): 316 – 318.

GURMUNSINGH (2018). Gene Therapy: A Medical Revolution. http://gurmunsingh.wixsite.com/genetherapy/about Accessed May 16**,** 2018.

HERSHEY, A. D. and CHASE, M. (1952). Independent functions of viral protein and nucleic acid in growth of bacteriophage. *Journal of General Physiology,* 36(1): 39 – 56.

HINGRA, D. (2018). Gene transfer techniques. PhD Seminar Presentation. Department of Plant Breeding and Genetics, Maharana Pratap University of Agriculture and Technology, Udaipur. https://www.slideshare.net/devhingra/gene-transfer-techniques-60176818/16 Accessed May 16, 2018

IMES, D. L., GEARY, L. A., GRAHN, R. A. and LYONS, L. A. (2006). Albinism in the domestic cat (*Felis catus*) is associated with a tyrosinase (TYR) mutation. *Animal Genetics,* 37(2): 175 – 178.

INNIS, M. A., MYAMBO, K. B., GELFAND, D. H. and BROW, M. A. (1988). DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proceedings of the National Academy of Sciences*, 85(24): 9436 – 9440.

ISENBARGER, T. A., FINNEY, M., RÍOS-VELÁZQUEZ, C., HANDELSMAN, J. and RUVKUN, G. (2008). Miniprimer PCR, a

new lens for viewing the microbial world. *Applied and Environmental Microbiology,* 74(3): 840 – 849.

JAENISCH, R. and BIRD, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics,* 33: 245 – 254.

JAROSLAV, P., MARKUS, G. E., ADAM, G., ALEXANDRA, F., SORIN, A., MASUMI, S., CHOUKRI, M., PIERRE, H. R. and SIGRID, N. (2002). Optimization of nonviral transfection: variables influencing liposome-mediated gene transfer in proliferating versus quiescent cells in culture and in vivo using a porcine restenosis model. *Journal of Molecular Medicine*, 80(11): 724 – 736.

KAMAU, S. W., HASSA, P. O., STEITZ, B., ALKE, P., HEINRICH, H., MARGARETHE, H. A., BRIGITTE, R. and MICHAEL, O. H. (2006). Enhancement of the efficiency of non-viral gene delivery by application of pulsed magnetic field *Nucleic Acids Research,* 34(5): 40 – 45.

KANEDA, Y. (2003). New vector innovation for drug delivery: development of fusigenic non-viral particles. *Current Drug Targets*, 4(8): 599 – 602.

KATO, N., NEMOTO, K., NAKANISHI, K., MORISHITA, R., KANEDA, Y., UENOYAMA, M., IKEDA, T. and FUJIKAWA, K. (2005). Non-viral HVJ (hemagglutinating virus of Japan) liposome-mediated retrograde gene transfer of human hepatocyte growth factor into rat nervous system promotes functional and histological recovery of the crushed nerve. *Neuroscience Research*, 52(4): 299 – 310.

KHAN, Z., POETTER, K. and PARK, D. J. (2008). Enhanced solid phase PCR: mechanisms to increase priming by solid support primers. *Analytical Biochemistry,* 375(2): 391 – 393.

KIM, Y. S., RHIM, H., CHOI, M. J., LIM, H. K. and CHOI, D. (2008). High-intensity focused ultrasound therapy: an overview for radiologists. *Korean Journal of Radiology*, 9(4): 291 – 302.

KUMARI, A., YADAV, S. K. and YADAV, S. C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B: Biointerfaces*, 75(1): 1 – 18.

LAWYER, F. C., STOFFEL, S., SAIKI, R. K., CHANG, S. Y., LANDRE, P. A., ABRAMSON, R. D. and GELFAND, D. H. (1993). High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5'to 3'exonuclease activity. *Genome Research*, 2(4): 275 – 287.

LEHRMAN, S. (1999). Virus treatment questioned after gene therapy death. *Nature*, 401(6753): 517–518.

LODISH, H., BERK, A., ZIPURSKY, L. S., MATSUDAIRA, P., BALTIMORE, D. and DARNELL, J. (2000). *Molecular Cell Biology.* 4th Edition, Scientific American Books, New York.

MAYEUX, R. (2005). Mapping the new frontier: complex genetic disorders. *The Journal of Clinical Investigation,* 115(6): 1404 – 1407.

MEHRDAD, H., AMIR, A. and PEDRAM, R. (2008). Hydrogel nanoparticles in drug delivery. *Advanced Drug Delivery Reviews*, 60(15): 1638 – 1649.

MIKLAVCIC, D., MIR, L. and VERNIER, P. (2010). Electroporation-based technologies and treatments. *Journal of Membrane Biology*, 236(1): 1 – 2.

MULLIS, K. (1990). The unusual origin of the polymerase chain reaction. *Scientific American,* 262(4): 64 – 65.

NABEL, E. G., GORDON, D., YANG, Z. Y., WU, B. Y., GAO, X., HUANG, L, XU, L., SAN, H., PLANTZ, G. E. and NABEL, G. J. (1992). Gene transfer *in vivo* with DNA-liposome complexes: lack of autoimmunity and gonadal localization. *Human Gene Therapy*, 3(6): 649 – 656.

NEUMANN, E., SCHAEFER-RIDDER, M., WANG, Y. and HOFSCHNEIDER, P. H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *European Molecular Biology Organization Journal,* 1(7):841 – 845.

NEWTON, C. R., GRAHAM, A., HEPTINSTALL, L. E., POWELL, S. J., SUMMERS, C., KALSHEKER, N., SMITH, J. C. and MARKHAM, A. F. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Research,* 17(7): 2503 – 2516.

ONGSIMEI (2016). *Types of Gene Therapy.* http://fbme.utm.my/ongsimei/ Accessed May 16, 2018.

PARK, D. J. (2004). 3'RACE LaNe: a simple and rapid fully nested PCR method to determine 3'-terminal cDNA sequence. *Biotechniques,* 36(4): 586 – 588.

PATHAK, A., PATNAIK, S. and GUPTA, K. C. (2009). Recent trends in non-viral vector-mediated gene delivery. *Biotechnology Journal,* 4(11): 1559 – 1572.

PAVLOV, A. R., PAVLOVA, N. V., KOZYAVKIN, S. A. and SLESAREV, A. I. (2004). Recent developments in the optimization of thermostable DNA polymerases for efficient applications. *Trends in Biotechnology*, 22(5): 253 – 260.

PAVLOV, A. R., PAVLOVA, N. V., KOZYAVKIN, S. A. and SLESAREV, A. I. (2006). Thermostable DNA polymerases for a wide spectrum of applications: comparison of a robust hybrid Topo Taq to other enzymes. Pages 241 – 257. *In:* KIELECZAWA, J. (Ed.). *DNA Sequencing II: Optimizing Preparation and Cleanup*. Jones and Bartlett Learning, Burlington, Massachusetts, United States.

PIERCE, K. E. and WANGH, L. J. (2007). Linear-after-the-exponential polymerase chain reaction and allied technologies Real-time detection strategies for rapid, reliable diagnosis from single cells. Pages 65 – 85. *In*: THORNHILL, A. (Ed.). *Methods in Molecular Medicine, Single Cell Diagnostics, Methods and Protocols.* Humana Press Incorporated, Totowa, New Jersey, USA.

PLANK, C., ANTON, M., RUDOLPH, C., ROSENECKER, J. and KRÖTZ, F. (2003a). Enhancing and targeting nucleic acid delivery by magnetic force. *Expert Opinion on Biological Therapy,* 3(5): 745 – 758.

PLANK, C., SCHILLINGER, U., SCHERER, F., BERGEMANN, C., ANTON, M., LAUSIER, J., KROTZ, F., REMY, J. S. and ROSENECKER, J. (2003b). The magnetofection method: using magnetic force to enhance gene delivery. *Biological Chemistry,* 384(5): 737 – 747.

ROBRISH, D. (1999). New technique permits faster, more accurate gene tailoring. *CNEWS Science.* <http://www.caldercup.com/CNEWSScience9907/26_gene.html> Accessed July 2012.

RYCHLIK, W., SPENCER, W. J. and RHOADS, R. E. (1990). Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acids Research,* 18(21): 6409 – 6412.

SAIKI, R., GELFAND, D., STOFFEL, S., SCHARF, S., HIGUCHI, R. HORN, G., MULLIS, K. and ERLICH, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* 239(4839): 487 – 491.

SAIKI, R., SCHARF, S., FALOONA, F., MULLIS, K., HORN, G., ERLICH, H. and ARNHEIM, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* 230(4732): 1350 – 1354.

SAKAGUCHI, N., KOJIMA, C., HARADA, A., KOIWAI, K., SHIMIZU, K., EMI, N. and KONO, K. (2008). Generation of highly potent nonviral gene vectors by complexation of lipoplexes and transferrin-bearing fusogenic polymer-modified liposomes in aqueous glucose solution. *Biomaterials*, 29(9): 1262 – 1272.

SAILS, A. D. (2004). *Applications of Real-Time PCR in Clinical Microbiology. Real-time PCR: an Essential Guide.* Horizon Bioscience, Norfolk, Caister Academic Press, United Kingdom.

SANGER, F., NICKLEN, S. and COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the*

United States of America, 74(12): 5463 – 5467.

SARKAR, G., KAPELNER, S. and SOMMER, S. (1990). Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Research,* 18(24): 7465 – 7470.

SCHERER, F., ANTON, M., SCHILLINGER, U., HENKE, J., BERGEMANN, C., KRUGER, A, GAUBACHER, B. and PLANK, C. (2002). Magnetofection: enhancing and targeting gene delivery by magnetic force *in vitro* and *in vivo*. *Gene Therapy,* 9(2): 102 – 109.

SMAGLIK, P. (2006). Chimeraplasty potential: as research advances, hopes rise, but efficiency and safety are still concerns. *Crigler-Najjar Syndrome, International Crigler-Najjar Meeting, Bertinoro (Forlì-Cesena, Italy),* 23 – 25 April, 2006. http://criglernajjar.altervista.org/fromthesc.htm Accessed May 16, 2018.

SONG, Y., GARAVENTA, G., BOTTERO, S., LEPRINI, A. E., PALLESTRINI, E., CASTELLO, E., PALLESTRINI, E. A. and HUANG, W. E. (2007). Ultrasound-mediated DNA transfer for bacteria. *Nucleic Acids Research,* 35(19): 1073 – 1080.

STEPHENSON, J. (1999). New method to repair faulty genes stirs interest in chimeraplasty technique. *Journal of American Medical Association,* 281(2): 119 – 121.

SUGAR, I. P. and NEUMANN, E. (1984). Stochastic model for electric field-induced membrane pores electroporation. *Biophysical Chemistry,* 19(3): 211 – 225.

THOMSON, K., CHEUNG, W., ELLIS, S., FEDERMAN, D., KAVNOUDIAS, H., LOADER-OLIVER, D., ROBERTS, S. and EVANS, P. (2011). Investigation of the safety of irreversible electroporation in humans. *Journal of Vascular and Interventional Radiology,* 2(5): 611 – 621.

VEISEH, O., KIEVIT, F. M., GUNN, J. W., RATNER, B. D. and ZHANG, M. (2009). A ligand-mediated nanovector for targeted gene delivery and transfection in cancer cells. *Biomaterials*, 30(4): 649 – 657.

VIJAYANATHAN, V., THOMAS, T. and THOMAS, T. J. (2002). DNA nanoparticles and development of DNA delivery vehicles for gene therapy. *Biochemistry*, 41(48): 14085 – 14094.

WOLF, Y. I., ROGOZIN, I. B., GRISHIN, N. V. and KOONIN, E. V. (2002). Genome trees and the tree of life. *Trends in Genetics,* 18(9): 472 – 479.

YANG, S. (2007). New medical technique punches holes in cells, could treat tumors. http://www.berkeley.edu/news/media/releases/2007/02/12_IRE.shtml. Accessed July 23, 2012.

YANG, Y. S., SUN, S. J., LIU, C. H., TSUANG, Y. H., CHEN, L. T., HONG, C. Y., YANG, H. C. and HORNG, H. E. (2008). *Ex vivo* magnetofection with magnetic nanoparticles: a novel platform for nonviral tissue engineering. *Artificial Organs*, 32(3): 195 – 204.

YAO, Q., CONG, L., CHANG, J. L., LI, K. X., YANG, G. X. and HE, G. Y. (2006). Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. *Journal of Experimental Botany,* 57(14): 3737 – 3746.

YOON, K., COLE-STRAUSS, A. and KMIEC, E. B. (1996). Targeted gene correction of episomal DNA in mammalian cells mediated by chimeric RNA-DNA oligonucleotide. *Proceedings of the National Academy of Science,* 93(5): 2071 – 2076.