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Review Article

Review of Gene Therapy Researches and Clinical Applications

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Abstract

The application of recombinant DNA technologies in human gene therapy was firstly raised several decades ago, with science advancement, the utilization of genetically modified techniques offered vast therapeutic benefits and potential to these patients with severe genetic diseases, such as muscular dystrophy, B-cell acute lymphoblastic leukemia, cystic fibrosis, etc. Here, the article reviews the history and progress of gene therapy for scientific researches and clinical practices, the strategies of gene therapy design, gene delivery approaches, and state-of-the-art genome editing techniques, CRISPR-Cas9, *et al*. Although multiple gene therapy products were successively approved by FDA to use in patients, the challenges of gene therapy in the clinical practice are still remaining. The ethical and safety issues of gene therapy is still a consideration in the clinical application, as a consequence, the long-term intervention of germline gene therapy need to be investigated and valuated properly before medical application. The common bottlenecks in gene therapy are still focusing on the level of gene delivery, and both nonviral and viral vector delivery approaches keep limitation in the clinical application and need to be further improved.

Introduction

Currently, about 6000 known human inherited diseases were found (Database from NIH National Human Genome Research Institute). These genetic diseases are caused by mutations or incorrect sequences in the normal form of the genes. Gene therapy is a technique to replace defective gene that causes a disease in an individual cells or tissues with a corrected gene, such as some severe hereditary diseases or cancer, etc. Gene therapy are also used for treatment of some lethal diseases through over-expressing functional gene, inactivating/knock-out a mutated gene, or regulating gene expression which is not functioning properly. Human Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder, researches show mainly point mutations, deletion, and duplication in the DMD gene cause prematurely truncated, dysfunctional dystrophins, a protein important for maintaining the stability of muscle-fiber membranes [1]. Most recently, Gene therapy with CRISPR/Cas9 system was used to restore the expression of the dystrophin gene in cells carrying dystrophin mutations that cause DMD. Researchers design single or multiplexed sgRNAs to restore the dystrophin reading frame by targeting the mutational hotspot at exons 45-55 and introducing shifts within exons or deleting one or more exons. As a result, human dystrophin expression is restored *in vitro* and *in vivo* [2]. Approximately 70 percent of the mutations in cystic fibrosis patients correspond to a specific deletion of three base pairs, which results in the loss of a phenylalanine residue at amino acid position 508 of the putative product of the cystic fibrosis gene [3]. Although clearance of viral vectors by immunal defense in lung airway sets up barriers of efficient gene delivery and expression of the therapeutic transgene, repeated nebulisation of nonviral CFTR gene therapy in patients with cystic fibrosis are in the clinical trails, it shows repeated administration of nonviral CFTR gene therapy holds potential for the further step along the path of translational cystic fibrosis gene therapy [4].

History and Progress of Gene Therapy

In past about 3 decades, a number of diseases have been studied or are ongoing with gene therapy, such as severe combined immuno-deficiencies (SCID), Hemophilia, Leber Congenital Amaurosis, Parkinson's disease, muscle dystrophy, cystic fibrosis, sickle cell anemia, cancer and HIV, *et al*. In 1990s, a clinical trail of gene therapy was initialed with retroviral-mediated the adenosine deaminase (ADA) gene transfer into the T cells of two children with severe combined immunodeficiency (ADA-SCID), after 4 years, it showed ADA gene expression in T cells persisted [5]. Later, gene therapy with hematopoietic stem cells, bone marrow and peripheral blood lymphocytes as vectors were used to deliver genes for correcting ADA-SCID hereditary diseases, which indicated safety and the efficacy of gene transfer [6,7]. Also, sickle cell disease (SCD) is successfully treated in mice with lentiviral-mediated gene therapy [8]. Subsequently, a tremendous amount of scientific researches related to gene therapy has been conducted to increase the understanding of the basic biology of the diseases, various gene therapy strategies and technologies were developed to treat diseases, also a series of guidelines and database were made to assist, monitor and assess the ethnic and safety issues of gene therapy by FDA and NIH (Guideline: Points to Consider in Human Somatic Cells Therapy and Gene Therapy, etc.; Database: ClinicalTrails.gov). Recently, gene therapy for some of lethal diseases has resulted into the successful outcomes in several clinical trails. The European medicines Agency approved the treatment of adult familial lipoprotein lipase deficiency patients with gene therapy medicine (Glybera). Subsequently, gene therapy products were successively approved to use in several different countries.

In 2003, it was approved to treat head and neck squamous cell carcinoma with gene therapy product (Gendicine) by the State Food and Drug Administration of China. In 2007, oncolytic adenovirus for treatment of late-stage refractory nasopharyngeal cancer and gamma-retrovirus vector expressing cytocidal cyclin G1 was approved by Philippine FDA. In 2013, a gene therapy product, plasmid vector expressing Vascular Endothelial Growth Factor gene was approved by Russian Ministry of Healthcare and Social Development to treat for peripheral arterial disease. In 2017, The FDA in Unite State approved the first three gene therapy products, CAR T-cell therapy (Kymriah) for treatment of certain B-cell acute lymphoblastic leukemia (ALL), the GALGT2 gene therapy program for treatment of DMD with dystrophin gene mutation, and AAV virus mediated gene therapy (Luxturna/voretigene neparvovec) for treatment of biallelic RPE65 mutation-associated retinal dystrophy. Especially, it is the first time that FDA approve AAV mediated gene therapy, compared with other viral vectors, the advantage of AAV as a safe gene therapy vehicle makes it to be feasible for clinical application. Furthermore, FDA announced 10-20 gene therapy drugs would be approved in one year by 2025. The most recently, the innovation and scientific advancement of the CRISPR-Cas9 mediated genome editing offers new possibilities for treatment of diseases that might be challenging or impossible to address with gene transfer technologies.

Strategies of Gene Therapy

According to the variant mechanisms of numerous genetic disorders, researchers developed versatile strategies of gene therapy, which can be classified as four types of strategies.

Over-Expression of a Functional Gene

Although it is difficult to know the side effects of over-expression of a functional gene to treat a disease, researchers were trying to over-express some existing genes due to their lack or dysfunction in diseases. In rat models of stroke and epilepsy, gene therapy of over-expressing hsp 72 with defective herpes simplex virus vectors improved neuron survival against focal cerebral ischemia and excitotoxin-induced seizures [9]. Also, over-expression of VEGF165 with recombinant Sendai virus (SeV) demonstrated the possible therapeutic treatment by delicate regulation of low VEGF165 expression in individuals with critical limb ischemia in murine model [10].

Homologous Recombination Based Knock-out/in

Development of Homologous recombination based knockout and knock-in technology make it possible to study the detail function of almost any genes. Combination of homologous recombination-based gene targeting and ES cells, numerous gene function and disease mechanisms were uncovered in past several decades. A number of researchers raised or developed different strategies for homologous recombination-based gene therapy. Sickle cell disease (SCD) can be corrected by homologous recombination with embryonic stem cells in mouse model [11]. Combination of nuclear transplantation and homologous recombination, it demonstrated immune-deficient Rag2-/- mouse can be repaired and restored normal function [12], etc. However, the low frequency of homologous recombination in mammalian cells and complicated technological programs limit its application for somatic gene therapy in human, also its safety and ethical issues keep the obstacles for its clinical applications on germline gene therapy.

siRNA, miRNA and shRNA-Based Regulation of Functional Gene Expression

Although siRNA, miRNA and shRNA have different molecular mechanisms and characterizations, all of them are working on the RNA level to regulate the RNA expression of the specific gene. RNA interefence (RNAi) was first experimentally documented in 1998 in Caenorhabditis elegans by Fire A, *et al*. [13]. Based on RNAi acting mechanism and principle, researchers designed synthetic small interfering RNA (siRNA) to regulate gene expression which can treat a wide range of diseases as therapeutic agents. siRNA is generally 21 to 25 bp in length which is designed to be homology with the specific mRNA sequences to knock-down its expression. Soutschek J, *et al*. [14] demonstrated chemically modified siRNA can silence an endogenous gene encoding Apopipoprotein B (ApoB) after intravenous injection which reduced total cholesterol level in the liver of mice. Short hairpin RNA (shRNA) are designed as sense and antisense sequences connected by a loop of unpaired nucleotides by synthesis *in vitro* or carry into cells with plasmid vectors which can produce functional siRNA by Dicer process in the cytoplasm. Koornneef A, *et al*. [15] showed knockdown of ApoB expression with AAV mediated shRNA reduce serum total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels in murine liver. miRNA are small (19-24 nt) non-coding endogenous RNA molecular exist in body which functions as negative regulators of coding gene mRNA expression by cleavage of specific gene mRNA or repressing gene translation. Usually miRNA affects genes responsible for cell proliferation, differentiation and apoptosis which may function as oncogenes or tumor suppressors. Numerous researches show miRNA may serve as a promising target for anti-cancer therapies. As potential therapeutic agents or targets, researchers can design artificial miRNA or miRNA tough decoys (TuDs) to enhance or inhibit specific miRNA expression, which can apply for these diseases caused by miRNA deregulation [16,17].

CRISPR/Cas Mediated Gene Editing

CRISPR/Cas system was discovered in bacteria as their adaptive immune response mechanisms against foreign DNA. Later, researchers reconstitute type II CRISPR-Cas9 system with a single guide RNA (sgRNA, the dual tracrRNA:crRNA engineered with complementary sequence of target gene) which functions like a global positioning system (GPS) to bring the endonuclease Cas9 protein into the targeted gene loci to create double strain break (DBS) on each strand of a DNA target site next to a PAM sequence, by this way, the targeted gene either can be silenced by creation of mutations or precisely perform error prone non homologue end joint (NHEJ). CRISPR/Cas9 mediated genome editing corrected the dystrophin gene (Dmd) mutation in a mdx mice model, which produced genetically mosaic animals containing 2 to 100% correction of the Dmd gene and greatly contribute to regenerating muscle [18]. Also, AAV-mediated local or systemic delivery of CRISPR/Cas9 endonucleases restored the Dmd reading frame, as a consequence, which partially recovered muscle functional deficiencies and generated a pool of endogenously corrected myogenic precursors in mdx mouse muscle [19]. Currently, uncover of crystal structure/domain architecture and understanding of subunit function of CRISPR/Cas9 allow researchers to design different variants of Cas9 systems for diverse purposes by modification of Cas9 and PAM sequences [20]. Cas9 that lacked nuclease activity (dCas9) fusing a transcriptional activation domain enable the CRISPR/Cas9 system to transcriptionally activate target genes within the native chromosomal context [21]. An Aspartate-to-Alanine (D10A) point mutation in the Ruvc catalytic domain of Cas9 nuclease (Cas9n) allows to nick rather than cleave DNA to yield single-strand breaks [22]. In addition, use of truncated gRNAs can reduce off-target

effects by 5000-fold or more [23]. In summary, Combination of DNA cleaving mechanism and the capacity for multiplexed target recognition in CRISPR/Cas9 system, it is feasible to develop the cost-effective and easy-to-use technology for precisely and efficiently targeting, editing, modifying genomic loci in a wide array of cells and organisms.

Methods of Gene Delivery with Gene Therapy

The success of gene therapy is greatly dependent on the DNA delivery into target cells. In past several decades, a number of DNA delivery methods are developed for gene therapy, including in nonviral and viral gene vectors delivery.

Nonviral Vector Gene Delivery

Vectors for gene therapy can be delivered into target cells or tissues with physical methods, such as, needle injection, electroporation, gene gun, ultrasound, hydrodynamic pressure and nanoparticles. In physical methods, the simplest and most safety way for administration of DNA is needle injection of naked DNA into target tissues or blood vessel. It is well known that nonviral gene delivery produces less immune response than viral gene delivery. However, the expression level and the area after injection of naked DNA are generally limited due to the rapid degradation by nucleases and clearance by the phagocyte system. As a consequence, it may need highly volume of DNA administration with nonviral gene delivery [24]. To improve the expression level of gene therapy vectors, various physical delivery methods have been developed. Electroporation is used for enhancing the ability of DNA permeation into cell membrane and uptake into cells after injection of naked DNA. Although electroporation can improve gene vector delivery and expression efficiency in various tissues, the complicated electroporation programs and parameters need intense and high professional labors to manipulate and optimize with the special medical devices [25]. Gene gun also can accelerate gene vectors delivery into tissues or cells by shooting DNA coated gold particles into the cells with high pressure gas. By this way, it allows DNA directly to penetrate through the cell membrane into the cytoplasm and the nucleus. Skin is one of the ideal targets to manipulate DNA transfer with gene gun [26,27]. Ultrasound employs ultrasonic wave to form short-lived (a few seconds) pores in plasma membrane which increases the DNA permeability into cell membrane after injection of naked DNA. Currently this technique is used for enhancing gene vector delivery to vascular cells, muscle and fetal mouse [28-30]. Hydrodynamic injection is simple and highly efficient delivery DNA into internal organs via injection of large volume of naked DNA solution with high speed into tail or other tissues vein, such as liver and limb muscles, *et al*. This simple and high efficient gene delivery both work well in small rodent and large research animal models, which will enable many gene therapy application in clinic [31,32]. Also gene vectors can be delivered into target cells mediated by some chemical carriers, such as cationic lipid, peptide and cationic polymer, which can form chemical-DNA complex to enhance gene expression via protection of DNA degradation or control of DNA release in specific cell types or cell layers [33]. Currently, this technology is still optimized for the researches and applications in the gene therapy field. Recently, it reported common cationic lipid nucleic acid transfection reagents also can potently deliver protein which used for protein-based genome editing *in vitro* and *in vivo* [34]. Due to the development of nano technology, a number of nanoparticles are optimized and used for the gene delivery based on the different chemical carriers, such as polymeric nanoparticles, lipid nanoparticles, magnetic nanoparticles, mesoporous silica nanoparticles, etc. According to the reports, these

nanoparticles display several desirable characterizations including low toxicity, well controlled and high gene delivery efficiency and multi-functionalities, etc [35-38].

Viral Vector Gene Delivery

Viral vector gene delivery use virus as vectors to deliver the target gene into cells or tissues to replace or modify the defective gene, currently main viral vectors for gene therapy are adenovirus, retrovirus, lentivirus, herpes simplex virus (HSV), and adeno-associated virus (AAV). Although some reports pointed out the risk of safety issues due to the host immune response to the viral vector system [39,40], numerous research reports and clinical practices have proved to be the most efficient methods of DNA transfer into host cells or tissues with viral vectors. A number of viral vector mediated gene therapy researches demonstrate these viral vector systems have unique advantages and limitations. Adenoviral vectors can efficiently deliver vectors to both dividing and non-dividing cell types with wide tissue tropisms, but immune clearance of infected cells often limits gene expression *in vivo*, also transient expression characterization make it not be good for treatment of genetic diseases [41]. In 1999, treatment of genetic disease, an inherited enzyme (OTC) deficiency, with adenovirus mediated gene therapy resulted into death of the patient, which speculated the cause of death is toxicity of high titer adenovirus and highly immunogenic [42]. Retroviral and lentiviral vector systems offer the advantages of large packaging capacity for gene delivery with cell and tissue specific tropism, also which can long-term express the target gene due to integrating into the genome of the infected cell [43]. However, both of retroviral and lentiviral vectors have risks to activate oncogenes to form tumor due to its insertional mutagenesis [44-46]. Although retroviral gene therapy cases are overall quite successful, a leukemia-like illness was developed in a SCID disease (T cells deficiency) treated with retroviral vector [47]. To harness the risk of oncogenicity, researchers developed integration-deficient lentiviral vectors with sustained and efficient transgene expression *in vivo*, which greatly reduces the risk of insertional mutagenesis [48]. HSV virus can deliver large amounts of exogenous DNA, a phase I/II clinical study of gene therapy based on retrovirus-mediated gene transfer of HSV type 1 thymidine kinase (HSV-1 TK) showed significant therapeutic responses for treatment of recurrent glioblastoma [49], however, cytotoxicity and maintenance of transgene expression remain as obstacles [50]. AAV virus can infect many non-dividing and dividing cell types for long-term gene expression without insertional mutagenesis, which makes AAV to be employed in gene therapy as a comparative safe and effective vector. Epidemiological studies have showed that 85% of healthy women are seropositive for AAV, which suggests AAV might have evolved an ideal relationship with its human host [51]. However, as an adverse effect, the presence of neutralizing antibodies (NAbs) after natural AAV infections inhibits their transfection efficiency in re-exposed subjects, which might affect AAV clinical application in the natural AAV infected population [52,53]. As disadvantages, engineered AAV has a limited DNA packaging capacity, which only can maximum pack 4.7~4.9kb DNA, also diversity of AAV serotypes and tropisms pose barriers to the universal application of AAV in gene therapy [54].

Types of Gene Therapy

Although Germline gene transfer or modification have widely researched in numerous genes in animal models, however, germline gene therapy practices in human are still facing the challenges of potential safety and efficacy issues. A number of somatic gene therapy for some lethal diseases have a great achievement

both in researches and in clinical practices, most recently, a few of gene therapy programs or drugs have been approved by FDA.

Germline Gene Therapy

Germline gene therapy results in genetic permanent changes inherited the next generations which have potential to offering a permanent therapeutic effect for these families with genetic diseases. The one way to manipulating germline therapy is to clean the defected gene in the male sperm cells with a genetic disease. In brief, a healthy gene is replaced to the defective one in each sperm-producing cell, and these replaced sperm-producing cells are screened and put into the testes for maturation and producing healthy sperm, finally in the laboratory, these healthy sperm was tested and used for fertilization. By this way, it is possible to eliminate the genetic disease permanently from a particular family. Also a defective gene can be modified in the female oocytes to produce healthy eggs for fertilization. Masahito Tachibana, *et al*. [55] investigated the feasibility of mtDNA replacement in human oocytes by spindle transfer, it shows spindle transfer embryos are capable of developing to blastocysts, although some oocytes displayed abnormal fertilization. The germline gene therapy has a potential benefit to permanently genetic modification of inherited lethal diseases, however, human germline gene therapy intervention is extremely difficult to study and evaluate experimentally, its medical perspective is limited due to the safety and ethical considerations.

Embryonic stem (ES) cells or induced pluripotent stem (iPS) cells also offer great potential for cell based gene therapy against genetic disorders, however, ES cells for gene therapy may need a patient-specific ES cells to eliminate or decrease the host immunorejection of cell transplantation, many ethical concerns are also involved in the clinical practices. As an option, iPS cell can be generated from an individual tissue cell with genetic manipulation through defined factors [56]. With iPS cell mediated gene therapy, it can contribute to genetic modification of the specialized function of any tissues and provide a genetic background match with the patient to decrease the likelihood of immunorejection. A genetic disorder, human DMD was treated via gene therapy with patient-derived iPS in a mouse model, which demonstrated chimeric mice with genetic corrected dystrophin expression in all examined tissues [57].

Somatic Gene Therapy

Due to ethnical considerations of germline gene therapy, most of diseases involved in genetic corrections are employing the strategy of somatic gene therapy which only affects the target cells. Somatic gene therapy can manipulate modified cells outside and then transplant back into body or the specific tissues (*ex vivo*), such as mesenchymal stem (MSC) cells, hematopoietic stem cell (HSC) and tissue-specific stem cells mediated gene therapy, etc. Bone marrow cells and peripheral blood lymphocytes mediated retroviral vectors gene therapy (*ex vivo*) was used for treatment of adenosine deaminase (ADA) deficiency in two patients, the result demonstrated the transferred ADA gene can express in T and B lymphocytes, marrow cells and granulocytes which resulted into normalization of the immune repertoire and restoration of cellular and humoral immunity after 2 years of treatment [7]. Also, HSC mediated gene therapy for two patients with adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID) showed engraftment of engineered HSCs with differentiation into multiple lineages, which result in increased lymphocyte counts, improved immune functions and lower toxic metabolites [6]. Subsequently, hematopoietic stem/progenitor

cells mediated lentiviral gene therapy for Wiskott-Aldrich syndrome (WAS) showed a positive therapeutic efficacy in three patients [58]. Currently, HSC mediated gene therapy is widely acceptable in the research and clinical application, although it provides an innovative treatment option for hematological disorders, long-term side effects are hard to predict and clinical experience remains limited.

Also gene therapy can be manipulated through directly transferring genetic agents into cells inside the patient's body (*in vivo*). Adenovirus-mediated herpes simplex virus thymidine kinase (HSV-tk) can treat brain tumor, gliomas by *in vivo* gene therapy [59]. With rat carotid artery balloon injury model, researches demonstrated endothelial cell nitric oxide synthase (ec-NOS) in the vessel wall was restored by the Sendai virus/liposome *in vivo* gene transfer, which shows potential to treatment of neointimal hyperplasia with *in vivo* ec-NOS gene transfer [60].

Prospect and Challenges of Gene Therapy

Most recently, development of CRISPR-Cas9 gene editing technology offers vast potential to manipulate gene therapy either in local somatic gene correction or in germline modification, which make the process of gene manipulation be simple, specific, high efficient and accurate in clinical application. Currently, although scientific and safety challenges do remain and emerge to be resolved, such as, improvement of gene vector delivery and gene editing efficiency, control of immune responses and potential adverse effects, evaluation of germline gene therapy intervention, decrease of off-target effects, etc., gene therapy has made a great progress from researches in model animals and clinical trails to produce measurable benefits in the clinic and commercialization. In 2017, The FDA in Unite State approved the first three gene therapy products, Kymriah for treatment of ALL, GALGT2 gene therapy for treatment of DMD, and Luxturna for treatment of retinal dystrophy. Given the rapid evolution and technological breakthrough in this field, it is reasonable that more and more gene therapy products and programs would be approved and commercialized.

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