Proceedings of the 2nd International Conference on Biological Engineering and Medical Science (ICBioMed 2022), Part II
DOI: 10.54254/2753-8818/4/20220649

Technologies in Gene Therapy

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Abstract. After the imagine of gene therapy been propose in 1967. and the successful case for the exogenous gene introduced into the mice. That's when scientists starting the experiment of gene therapy. This review focus on the history of gene therapy, and two important technology about the gene therapy: CRISPR/Cas9 and RNA inference. Talking the theory of them, also compared with the traditional treatment and gene therapy to the same disease. And have a discussion of the status and the application prospect of the gene therapy.

Keywords: gene, therapy, CRISPR/Cas9

1. Introduction

Gene therapy is to introduce specific engineered genes into the patient cell, as a treatment for disease that cause by genetic anomalies. In 1990, U.S. FDA firstly approved the clinical trial of gene therapy. A four-year-old girl which suffered the Aadenosine deaminase deficiency (ADA), injunction the normal gene segment which use to produce the Aadenosine deaminase. Aadenosine deaminase is an autosomal recessive disorder. Loss of the Aadenosine deaminase will cause the accumulation of metabolite such as adenosine, deoxyadenosine, particularly the deoxyadenosine triphosphate, these will inhibit the synthesis of DNA, resulting in the dead of the T-cell and B-cell dysfunction. That will cause the serious SCID, the patients that have the ADA even cannot survive infancy. During this treatment, the patient been infusion periodic the culture-expanded T-cell, which been genetically correct by inserting the normal ADA gene by using the retrovirus, in order to treat the immunocompetence of the patient.

However, gene therapy also be difficult to control and predict. An example in 1999. Jesse Gelsingers attend in a clinical trial in order to treat the ornithine transcarboxylase deficiency. For the patient who have OTC deficiency, because of the mutation of gene, the OTC's enzyme activity decreases or disappear. The process of ornithine and carbamoyl phosphate combine to produce the citrulline be impeded, resulting in an abnormal concentration of ammonia in the blood. But that patient who firstly attend the treatment of gene therapy was died because of the serious immunoreaction. The gene delivery vector to keep away from the immune system, otherwise the immunoreaction will cause the serious disease or even be died. That matter causes the great blow of gene therapy, because it is the first deaths cause by gene therapy. After it, the treatment for OTC deficiency still be the liver transplantation or low protein diet with nitrogen scavenger. Also, there will have a problem for destruction of the important gene in the target cell during the treatment. That because the gene may cannot insert in the correct part of gene, and damage another gene which is normal.

This review focus on two technologies in gene therapy, CRISPR/Cas9 and RNA inference. In the review we will talking about how technology be found, the mechanism and the clinic treatment for them.

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DOI: 10.54254/2753-8818/4/20220649

Using the results to compare with the results of conventional treatment in order to show the advantage of gene therapy, and talking about the barriers and the future expectation.

2. CRISPR/Cas9

2.1. History

CRISPR firstly been found from Escherichia coli in 1987, but it been named until 2002. It is common to find the similar structure of CRISPR in the bacteria and archaea genomes. CRISPR loci be consist of direct repeat, them be separate by the spacers that are stretches of variable sequences.

Compared with organism, there is no immune system in bacterial and archaea. In order to defend the virus infection, these microorganisms need other defense mechanisms. Mostly in archaea and many bacteria, CRISPR, which is the clustered regularly interspaced short palindromic repeats from peculiar genetic loci. The way is to target nucleic acid in a sequence-specific manner, for acquiring the immune of virus and plasmids [1-2].

2.2. Mechanism

CRISPR-Cas9 is actually the acquired immune system in the bacteria or archaea that used to defend the virus. CRISPR will firstly detect the exotic DNA that virus injunction into the cell, after the detection, short piece of the viral DNA section allows to integration into the CRISPR locus. That is the first step of bacteria to get the acquired immunity. The second step which is called the CRISPR RNA biogenesis, these CRISPR sequences be transcript to the RNA to form interfering or interference complex with the protein that encoded by the CAS gene. Then the CRISPR RNA base pairing the viral DNA [3].

Cas9 been found in the Streptococcus pyogenes, which is important in the nature CRISPR-Cas9 system. In CRISPR-Cas9, CRISPR RNA (crRNA) will combine with another RNA which called tracer and form to a structure which can recruits the Cas9 protein. CRISPR RNA can position the DNA, then part of it can base pair with one strand of double helical DNA, then the Cas9 can generate the double strand break of the exotic DNA. In the nature, both of the RNA and the Cas9 protein is indispensability. However, during the test been programmed by human, they combine the crRNA and the tracer RNA, to form a single guide RNA (sgRNA) to replace two of the RNA. They design an experiment to show sgRNA be correctly programmed to cut the target gene by incubate a plasmid (circular DNA molecule) in two different restriction(cutting)enzyme, one enzyme named ‘sall’, another enzyme is RNA-guided Cas9. After this incubation reaction, they use the agarose gel to separate the DNA, and it is clearly to show the DNA be cut comply with the design. This experiment showed DNA cutting enzyme can be programmed.

For the DNA double strands be break, the cell will try to repair itself by two pathways. One is NHEJ, which means the end of the DNA will ligated together, but during the ligation, it will accompanied with the losing or adding some small section in the break site of DNA. Another way of repair, called homology directed repair. It is to set a donor DNA that can match with the break strand, and then into the genome at the site, then its information can add into the genome. Some genetic defect disease can be treated by finding the mutation section, break them and introduce the DNA that can fix a mutation or generate a mutation [4].

Compare with the ZFN and TALEN, CRISPR/Cas9 is more operable, it even can edit all of the genes because one out of every eight bases can be found in genome that can be edit by the CRISPR/Cas9 in theory. The controllability of CRISPR/Cas9 is better. For example, by modifying for the Cas9 protein to only cut one strand of DNA can avoid the risk of the mutation of chromosome causing by the cut of the double strand of DNA. Because CRISPR/Cas9 is an RNA programmed protein, by changing the sequence of the guide RNA associate with Cas9 protein, any site of DNA can be generating to break by using single protein, instead of ZFN and TALEN which are rely on the protein-based recognition of DNA. That means is CRISPR/Cas9 is a system that is simple enough to use that anybody with basic molecular biology training can take of this system to do genome engineering

In CRISPR/Cas9 system, CRISPR/Cas9 be guided to interrogate the genome by the single guide

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RNA (sgRNA) which is a short RNA who can matching the target DNA fragment. But single guide RNA can tolerate certain mismatches to the target section of the DNA in order to promote undesired off-target mutagenesis. So, the limitation of CRISPR/Cas9 is how to eliminate the off-target effects. There are strategies for solving the off-target effect. (1) Design the single guide RNA with specificity. (2) Found the novel Cas9 protein with higher specificity. For example, a novel Cas9 protein (saCas9) was found in staphylococcus aureus, the saCas9 protein has ability to edit gene and have specificity be shown by in vitro experiment.

2.3. Example

Hemophilia be seen as a inherit disease which cause by the loss of blood coagulation factor. It is classified into two type: Hemophilia A and Hemophilia B. The Clinical manifestations for hemophilia like mucocutaneous hemorrhage, hemarthrosis, hematoma. The common treatment for hemophilia is uses the alternative medicine. For example, to treat hemophilia A can use fresh frozen plasm to provide the losing blood coagulation factor Hemophilia B cause by the loss of blood coagulation factor Ⅺ. Blood coagulation factor Ⅺ is formed by 416 amino acid residues. During the activation, a part of peptide will release, and two of the peptides will connect by disulfide bond [5]. The heavy chain the catalytic active site of enzyme, and the light chain will combine with the phospholipid that provide by platelet. The Ⅸa factor will combine with the Ⅷ factor when there are copper (Ⅱ) ion and phospholipid.

Make Ⅹ factor be activated to Ⅹa factor. Then the Ⅹa factor will form to be a compound with phospholipid, copper (Ⅱ) ion and Ⅴ factor to form into a compound. That compound will help thrombin be activated into thrombin. Hemophilia B is cause by the loss of Ⅺ factor.

The traditional treatment for hemophilia B is to supplement the losing blood coagulation factor by injunction the fresh blood and the fresh frozen blood. But it is difficult to let the blood coagulation factor to reach the effective hemostatic concentration. But there is also the risk of hemolysis and virus injection. The more efficient way is to injection of cryoprecipitation, because there is higher concentration of blood coagulation factor compare with the fresh frozen blood, so it will have the effect on the treatment of the patients with mild and moderate hemophilia A, but it will cause the produce of Ⅷ and the Ⅸ inhibitor, that will let the treatment be more difficult with the high occurrence rate about 10% [6]. Another ways which is more efficient than other is to injunction the virus inactivity blood-borne FⅧ preparations and the virus inactivity blood-borne FⅨ preparations, the former for patient who have hemophilia A, the latter for patient who have hemophilia B.

But all the treatment be mentioned is the short-run treatment. Scientists are trying to finding a treatment that can achieve the long-run correction of hemophilia. Because hemophilia B is a monogenic inherit disease, it is one of the most primary targets in the gene therapy. Gene therapy that using the recombinant adeno-associated viral (rAAV) to attained long-term disease correction is already been achieved. But it only been showed the efficiency in the adult patient for liver-directed gene transfer, not enough clinical trials in young patient and pediatric. Because compared with the quiescent adult livers, in the non-adult patient the transferred epitomal genes will dilute from the cell division during the growth because of vector dilution and the loss of transduced cell population. It can be showed that the need for young patient and pediatric patient to achieve the long-term gene expression is not be satisfy [7]. They turn to the gene editing, because it maybe allows for corrective genes be permanent incorporation into the patient chromosome for life time. The integration may can counteract the loss of transduced cell populations if progenitor or stem cells genetically incorporate the corrective transgene and are not eliminated.

Targetable editing-base genomic integration approaches allow the addition of corrective gene or in situ correction of the endogenous defective gene. This approach may be can circumvent the loss of gene expression with epitomal persistence-based vectors, and useful for in vivo somatic therapy. Some scientist tries to use the lentiviral vectors (LVs) integrate with viral integrases to let sustained gene be expression sustained in the pediatric patients who have severely immunodeficient. However, this

DOI: 10.54254/2753-8818/4/20220649

integration will perturb the surrounding genomic environments because it will occur promiscuously throughout the genome of target cell with a preference for transcriptionally active regions. It is highly desirable to use the method like targeting integration to specific well-characterized locus (safe harbor), or to endogenous disease loci. Gene editing is a plausible strategy to treat models of inherit disease, including the neonatal hemophilia B. It is been show by the gene delivery methods which use the rAAVs. In fact, rAAV is currently the preferred platform for liver-directed therapies to correct monogenic disease.

However, after administration in prenatal and neonatal animals may cause the residue of rAAV carriers and affect residual gene expression levels in developing organisms [8]. Likewise, the application of rAAV vectors will also be constrain in some contexts because of the risk of random integration of rAAV vector sequences and insertion of vector genomes at editing target sites.

Compared with the rAAV vector, the mediate editing-based integration use Adenovirus vectors has not been explored to the same degree as rAAV. The Adenovirus vectors can be useful for elaborate gene editing strategies and lager genes' integration depend on its large packaging capacity. The difference will rAAV vectors is Adenovirus vectors is difficult to be integrate into the host genomes. However, every virally transduced cell maybe encounters vector silencing, immune-mediated elimination, cell death or high cell turnover, can causing the loss of gene expression. Gene delivery methods which use the Adenovirus vectors can accomplish disease correction for editing-based integration with reduced genotoxic risk. Scientists use Adenoviral delivery of CRISPR/Cas9 to mediate targeted gene integration in vivo. It may have several advantages, for example the repair accuracy and the size of constructs may is greater than other approaches. They use this method to mediate corrective gene knock-in at the ROSA26 safe harbor locus, and found a single injection of adenoviral vectors achieved mFIX cDNA knock-in at the ROSA26 safe harbor and long-term phenotypic correction of the Hemophilia B bleeding diathesis. It is demonstrating that adenoviral vectors are capable of targeted gene integration and long-term correction of an inherit disease in juvenile mice.

3. RNA inference

RNA inference firstly been found in Caenorhabditis elegans, they use the small RNA molecule to target precisely, and cause the gene silence. RNA inference has the ability to specifically and potently knock down the expression of disease-causing genes of known sequence, also it is theoretically amenable to rapid targeting even in newly discovered pathogens.

Gene silence is one of the most important way for eukaryote cell to regulate the expression of the gene. Which is the mechanism for double-strands RNA (dsRNA) induced to recognize and eliminate the mistake RNA. It is often the reaction process such as the deacetylation of lysine residues in the N-terminal domain of histones and the Hypermethylation, resulting in the corresponding section of gene loss the activity of the transcription and been gene silence [9].

3.1. Mechanism

The small RNA molecules are mostly been small inference RNA (siRNA) and micro RNA (miRNA). During the exogenous gene such as the viral gene and the gene be delivered into the cell experimentally, been transcription in the cell and their gene integrate into the host genome, during the transcription, it will often produce some double-strands RNA. The RNA endonuclease Dicer will cut the double-strands RNA into the small segment RNA with the specially length and structure(21-23bp), which is the small inference RNA. Then the two strands of small inference RNA been separate by helicase, one strand is the sense strand, another is the antisense strand, the antisense strand which is also called guide strand, that strand will combine with the argonaut protein [10]. The endonuclease Argonaute 2 (AGO2) is responsible for the cleavage mechanism of RNA-induced silencing complex (RISC). It is the only member which observed catalytic activity in the argonaure subfamily of protein in mammalian cell [11].

There two mainly domain in the argonaunt protein, one is PAZ, another is PIWI, PAZ is the bonding site for siRNA or miRNA. The structure of PIWI domain is similar to the Ribonuclease H (RNase H) which is form from five Lamella structure that is surrounding by the Helical structure. The PIWI domain

DOI: 10.54254/2753-8818/4/20220649

have the similar function with RNase H, which can be used to cut the RNA strand in the dsRNA which is combine with the siRNA and RNA. But RNase H is been used to hydrolysis specifically the RNA in DNA-RNA hybrids. RNA induced silencing complex (RISC) been form by the siRNA or miRNA, argonaut protein and other proteins [12]. The targeting of RISC and the messenger RNA is precise because it depends on the base pairing with the siRNA and the messenger RNA. After bound, the argonaut protein will catalyzed the cleavage of the messenger RNA, and then the messenger RNA will be degraded.

The passenger strand of the double-strand siRNA or the AGO2-mediated cleavage of the sense strand will then generate into the single-stranded antisense strand which can combine with the RNA-induced silencing complex, and guide it to complementary sequences in target mRNA.

There will be the perfect or near-perfect Watson-Crick base pairing between the mRNA and guide or antisense strand of siRNA. So that the siRNA can position the target gene by pair bonding with the target section. The cleavage of targeted mRNA will happen between base 10 and 11 relative to the 5'end of the siRNA antisense strand. The mRNA transcript will then be degraded by cellular exonucleases.

3.2. Example

There is an increasing interest in developing novel drugs for the disease (neurological disorders viral infection or cancer) depend on harnessing the gene silencing activity of double-stranded RNA (dsRNA). Especially the drugs base on small inference RNA (siRNA), compared with other small molecule or protein-based drugs, the small inference RNA based drugs has high specificity and reduced toxicity. Even almost every disease cause by gain-of-function genetic lesion of overexpression of diseases causing gene is a potential target for RNA-inference-based therapeutics, this concept been proof by several in vitro and in vivo studies, which is also one of the important reasons for RNA-inference-based drugs has been moved rapidly from laboratory to the clinic [13]. Some animal models have already shown that how effective the RNA-inference-based drugs can be in the various diseases' treatments, including the viral hepatitis, hypercholesterolemia, Huntington's' disease and cancer.

Overexpression of lipogenic enzymes can cause many common diseases, such as obesity and diabetes. Some scientists use the key lipid metabolized enzymes which found in adipose tissue to be the target of drugs for obesity. Likewise, it is also a common characteristic of many cancers, including Prostatic cancer (PCa), mammary cancer and Hepatocellular Carcinoma (HCC). Because there is an increasingly recognized that the enhanced expression of lipogenic enzymes can be a common characteristic of a wide variety of tumors. Also, there was a study that for nearly all nonmalignant adult tissue the lipogenesis is low, in comparison in the tumor, where the lipogenesis is high. However, that recognized led to the exploration of use the endogenous lipogenesis as a target for prevention or treatment of cancer. Up to now, most of the attempts is focus on the fatty acid synthase [14].

Fatty acid synthase (FAS) is the key lipogenic enzyme which can catalyze the terminal steps in the de novo biosynthesis of long-chain fatty acid. The reason for the increasingly recognized that enhance expression of fatty acid synthase is a common reason for most of the tumors is that the overexpression of fatty acid synthase has been found in many earliest stages of tumor development [15]. The overexpression of fatty acid synthase will aggravate as the tumor progresses in wide variety of tumor types. For let the fatty acid synthase as the target for prevention and treatment the cancer, compared with using the chemical inhibitors of fatty acid synthase, including Cerulenin, Orlistat, EGGG and c75. people prefer to use the much more selective approaches such as RNA inference (RNAi) with small inference RNA (siRNA) targeting the fatty acid synthase to arrest the growth of tumor cell or make the tumor cell death [16].

However, the accumulation of the toxin intermediate malonyl-CoA will cause the cytotoxicity induced by fatty acid synthase inhibition. In order to gain more cytotoxicity mechanism induced by fatty acid synthase inhibition, also to explore whether other lipogenic enzymes also can have the potential to be the target for cancer intervention. Scientists compared the selectively knocked down fatty acid synthase expression in prostate cancer cells using RNA inference with the effect with those of the ACC-α RNA inference. They found that the down-regulation of both fatty acid synthase and ACC-α will result

DOI: 10.54254/2753-8818/4/20220649

in the inhibition of the proliferation of LNCaP, and cause the apoptosis of caspase-mediated. After the transfection with ACC-α siRNA, there were no accumulation of malonyl-CoA been observed, on the contrary, there was 9 times of the intracellular malonyl-CoA-level after the transfection with fatty acid synthase siRNA. That show the facts that ACC-α is also involved in fatty acid elongation, fatty acid synthase would not affect the synthesis of cholesterol, but ACC-α RNA inference will slightly reduce cholesterol synthesis.

4. Conclusion

RNA inference has been considered as a therapeutic approach, because there are inherent difficulties for using the conventional approaches to blocking many desirable targets. However, although there is immense potential to use RNA inference as a therapeutic, many physiological obstacles impede the successful and efficient delivery. RNA inference can circumvent many of the problem which will happen in the using of small-molecule inhibitors. For example, a small-molecule inhibitor of a specific kinase will not affect the oncogenic function unrelated to kinase. RNA inference is highly attractive as a therapeutic approach been bolstered by a report of systemic small interfering RNA (siRNA) delivery into human tumor. That report showed how to use the siRNA to target N2 subunit od ribonucleotide reductase (RRM2) which is difficult to inhibit by using conventional approaches. However, to introduce the RNA-inference-based therapies into the clinic scientists still have to overcome several hurdles, including efficient and safe system delivery and avoidance of undesirable off-target effects. Governing the heterogeneity of tumors, the resistance for RNA inference must be inevitable. Some cancers may be inherently resistant to certain types of RNA inference because of the reason including ethnicity, altered RNA inference processing machinery and somatic mutation. But these problems can be solved by understanding the resistance mechanisms in cancer. Another key challenge for developing RNA-inference-based therapies is to solve the issue of whether siRNAs can be effectively delivered to the target cell and appropriate targets can be identified to destroy the metastatic population. For cancer, the main problem is to targeting metastatic cells that have spread from the original tumor. For the neurodegenerative disease such as Amyotrophic Lateral Sclerosis (ALS), the main challenge for them is delivery of RNA inference to specific cells in the nervous system. In experimental animal models, but there may have the requirement for multiple siRNA.

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