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The Key Technique in the mRNA Vaccine Therapy

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Abstract: mRNA-based gene therapy drugs and preventive vaccines have become a hot topic of development. The technical principle is that mRNA delivered to the body cells can be expressed through the translation mechanism of host cells to produce therapeutic proteins or immunogenic proteins, which have therapeutic effects on the body or stimulate the body to produce immune effects. mRNA vaccines have many advantages over traditional vaccines, such as their safety, flexibility, rapid production and low cost. mRNA vaccines are therefore proposed as a new frontier in vaccination. in this review, the main key technologies based on mRNA drug design, such as in vitro transcription (IVT), nucleotide and cap and tail structure modification, were reviewed, followed by a brief introduction to mRNA purification methods and delivery systems. Several novel mRNA drugs are also briefly described. Finally, the latest research directions and possible challenges of mRNA are mentioned, aiming to provide support for the development of mRNA gene therapy drugs and vaccines.

Keywords: mRNA Vaccine; Lipid Nanoparticles (LNP); Cancer Treatment; Infectious Diseases; Delivery; In Vitro Transcription; mRNA Modification.

1. Introduction

In 1961, Brenner et al. proposed the carrier of genetic information between DNA and protein -mRNA[1]. Due to its instability, the idea of applying to disease treatment has not been verified. It was not until 1990, when Wolff et al. expressed mRNA transcribed in vitro in mouse skeletal muscle for the first time, that the feasibility of mRNA drugs became apparent[2]. In recent years, the research on mRNA vaccine has been carried out, but it has not been applied to human body. The success of SARS-COV-2 vaccine MRNA-1273 and BNT162b2 has made people realize the advantages of mRNA drug therapy and the broad development prospect in the future.

mRNA vaccines have huge advantages. Most mRNA vaccines are produced and purified in a similar way, and when different proteins are needed, the mRNA sequence is theoretically only changed, which is important in terms of time and economic cost savings, especially in response to some global health emergencies such as COVID-19. In addition, it is also useful for viruses that can mutate, as RNA viruses like COVID-19 are prone to mutating, rendering existing vaccines ineffective. But the similarity in the manufacturing process has allowed pharmaceutical companies to quickly develop similar mRNA vaccines. In addition, in vitro transcription also makes it easier to produce mRNA vaccines. At the same time, mRNA vaccines are safer than DNa-based nucleotide vaccines. Except for the rare recombination of single-stranded RNA molecules, mRNA vaccines do not integrate into the host genome, and mRNA is expressed in the cytoplasm, thus avoiding possible genetic contamination. In addition, mRNA vaccines have autogenous properties that induce the body to produce cytokines that activate a strong and long-lasting immune response compared to protein-based vaccines, which require additional adjuvants to achieve the same effect.

In the long run, in addition to COVID-19 vaccine, mRNA technology has gradually matured in the fields of tumor vaccine, gene editing, CAR T cell therapy, protein replacement therapy and other infectious disease preventive vaccines, and products have emerged one after another. mRNA has become a hot research and development spot in the field of biomedicine in recent years.

2. mRNA Modification and Synthesis

Exogenous mRNA, like endogenous mRNA, needs to be sequenced and modified in order to function in the human body. For example, cap structure and tail structure are critical for the stability of mRNA in the human body, while the design of the open reading frame (ORF) and UTR affects the efficiency of mRNA translation. There are reports that mRNA can cause the body to produce type I interferon and cytokines, which can induce immune rejection and reduce the effectiveness of drugs. In addition, the self-adjuvant effect makes mRNA have strong immunogenicity, which will strengthen the immune response that stimulates the body to produce similar to the rejection of RNA viruses. Therefore, it is necessary to find feasible measures to reduce the immunogenicity of gene therapy products.

2.1. DNA Design

The target DNA for transcription is often prepared by plasmids. Plasmid DNA should be designed to include replication start sites, promoters, 5 'UTR, ORF, 3' UTR, polyclonal sites, resistance genes, and in most cases poly (A) tail coding sequences. Before synthesizing the target gene sequence, selecting appropriate 5 ', 3 'UTR coding sequences and poly (A) tail coding sequences to design at both ends of the target gene will be conducive to mRNA stability and expression. In addition, when designing DNA sequences, it is often necessary to analyze and optimize ORF coders to obtain the optimal coding efficiency.

2.1.1. UTR Design

UTR affects mRNA decay and translation efficiency. The 5 'UTR sequence plays a key role in protein expression, and the possible mechanism is that it affects the recognition and binding of ribosome and mRNA at the translation initiation

stage, thus affecting the translation efficiency of mRNA, including ribosome recruitment, scanning and the selection of start codon. Some unique sequences can be added to the 5 'end to improve the translation efficiency and stability of mRNA. Compared with the 5 'UTR, the 3' UTR mainly affects the half-life of mRNA, and it is a concentrated region of unstable factors. When designing this region, increasing the proportion of stable sequences, such as AU and GU rich regions, can effectively improve the stability of mRNA. Studies have shown that the tandem application of 3 'UTR containing stable sequences significantly improves mRNA stability and translation efficiency.

2.1.2. ORF Design

The codon sequence in ORF affects the mRNA translation efficiency. Efficient translation of mRNA is not always beneficial, and some proteins require relatively low translation efficiency for structural stability. Therefore, the translation efficiency of mRNA can be adjusted by adjusting the frequency of rare codons in ORF. Codon optimization has been proved to remarkably enhance protein expression by adding frequent codons and codons with higher tRNA abundances. In addition, mRNA stability and protein expression in vivo can be improved by enriching guanine and cytosine contents.

2.2. mRNA in Vitro Transcription

The technology of mRNA transcription in vitro has been relatively mature. Phage RNA polymerase uses constructed linearized DNA as template, nucleotides and modified nucleotides as raw materials to initiate transcription and produce mRNA precursors. At present, the most popular phage RNA polymerases are T3, T7 and SP6RNA polymerases, among which T7RNA polymerase is the most commonly used.

2.3. Cap

Most mature mrnas of eukaryotes also have a cap structure ending in 7-methylguanosine. The 5 'cap plays an important role in improving mRNA function by eliminating free phosphate groups in the mRNA sequence, thereby significantly enhancing mRNA stability, enabling ribosomes to recognize the start of mRNA, and improving translation efficiency by binding to eukaryotic translation initiation factor 4E (eIF4E). The structure of the 5 'end hat has three types, divided into type 0, Type I and Type II. Type 0 hat refers to the end of the nucleotide ribose without methylation, type I hat refers to the end of the nucleotide ribose is methylated under the catalysis of the enzyme, type II hat refers to the end of the nucleotide ribose is methylated. In 2014, KUMAR et al. tested the ability of pattern recognition receptors to recognize capped mRNA in three different ways, including cap0, cap1 and uncapped mRNA, and found that cap1 capped mRNA could still translate into protein after being recognized by pattern recognition receptors. However, cap0 and uncapped mRNA cannot translate and produce proteins after being recognized by pattern recognition receptors[3], and the function of type II hat structure has not been explored. Therefore, selecting the appropriate type I hat mode can avoid inhibiting the translation efficiency of mRNA due to strong immune response. There are two kinds of mRNA cap modification techniques, enzyme based cap method and cap analogue cotranscription method. Enzyme-based capping method refers to in vitro transcription through methyltransferase reaction, such as vaccinia virus capping enzyme developed by MARTIN et al. 1975 binding 2 '-O-methyltransferase to produce cap1[4]. However, this method also has drawbacks, the use of vaccinia virus capping enzyme is expensive, enzyme production cost is high. In addition, the enzyme cap introduces additional proteins, resulting in the need for multiple purification in the preparation.At present, hat analogs cotranscription method is more used, that is, hat analogs are directly transcribed in vitro to generate mRNA with hat structure. However, studies have found that conventional hat analogues bind mRNA sequences in reverse, resulting in the formation of mRNA isomers, which affect translation efficiency. To avoid the reverse infiltration of 5 'hats, an anti-reverse cap analogs (ARCA) has been developed and found to be more efficient in translation than the conventional cap approach. Research on ACRA has been ongoing in recent years. For example, ARCA-based thiophosphate modification can improve mRNA translation efficiency by increasing mRNA affinity to eIF4E. In 2018, another co-transcription capping method called "CleanCap" was developed, which uses an initial capping trimer to produce a naturally occurring 5 'end-cap structure with a capping efficiency of 90% to 99%, and the most mRNA companies are currently using this capping strategy.

2.4. Tail

Poly (A) tail can promote mRNA translation efficiency and enhance mRNA stability by reducing RNA exonuclease activity. In addition, poly (A) binding protein (PABP) can help to form mRNA ring structure and improve mRNA translation efficiency by binding the translation initiation factors elF4G and elF4E to the 5-terminal cap. Conversely, PABP can also be combined with adenosine compound, involved in translation of microRNA mediated inhibition process[5]. The dual role of PABP in mRNA translation suggests that poly (A) design should be moderate, not too long or too short. In 2006, HoltKamp et al. showed that A poly (A) tail using approximately 120 nucleotides in DC has better mRNA translation efficiency and stability compared to a shorter tail[6]. However, Lima et al. showed that mrnas with high translation efficiency actually have shorter poly (A) sequences (33-34 nucleotides)[5]. In conclusion, appropriate poly (A) length should be selected for different cell types to achieve the best mRNA translation efficiency. In general, there are two ways to add poly (A) structures, pre-transcriptional and post-transcriptional. By designing the corresponding poly (T) sequence in the DNA template, the mRNA obtained by transcription has the tail structure of specific length directly. Post-transcription coaxing is the addition of poly (A) structure by polymerase polymerase catalyzing the polymerization of adenylate in the RNA tail. Compared with the two methods, post-transcription coaxing usually results in A mixture of poly (A) structures of different lengths, while pre-transcription coaxing results in a poly (A) structure of a certain length with fewer enzyme use and removal steps, so it is more advantageous.

2.5. Nucleotide Modification

Nucleotide modification is considered the most important breakthrough in the function of mRNA drugs in the human body. Unmodified mRNA is recognized by body leading to innate immune activation, resulting in significantly reduced mRNA translation efficiency. Some studies have shown that using modified nucleotides such as pseudouridine instead of original nucleotides can effectively resist RNA enzymatic

hydrolysis and improve translation efficiency and stability. At the same time, some studies have shown that the use of modified nucleotides such as thiouridine and 5-methylcytidine can reduce immunogenicity. In addition, some studies have also mentioned that the use of methylpseuduridine is better than pseuduridine. The currently approved BNT162b2 vaccine from BioNTech and the mRNA-1273 vaccine from Moderna both use methylpseuduridine as a modified nucleotide.

3. mRNA Purification

It has been confirmed that mRNA produced by phage RNA polymerase transcription in vitro contains a variety of impurities, such as short RNA generated by transcription interruption, oligodeoxynucleotides, dsRNA catalyzed by RNA-dependent RNA polymerase, and proteins, which may lead to low levels of immune rejection. Efficient mRNA purification can not only remove the impurity RNA contamination that causes immune rejection, but also improve the efficiency of mRNA translation. Licl-ethanol precipitation method is commonly used to remove impurities, LiCl precipitation has greater advantages than other RNA precipitation methods, and its efficiency is higher to precipitate DNA and protein, and then the target mRNA was separated from other mRNA impurities in the system by high performance liquid chromatography (HPLC).

4. mRNA Drug Delivery System

Exogenous mRNA must reach the cytoplasm through the cell membrane in order to translate the protein. Because mRNA molecules are large and negatively charged, they repel the anionic lipid bilayer on the cell membrane and thus cannot effectively enter the cell to perform functions. In addition, mRNA is easily engulfed by immune cells in the body or degraded by nucleases in the environment. An efficient mRNA delivery system is therefore critical to achieving therapeutic relevance. At present, there are several main delivery methods of mRNA, among which nano lipid particles are the most promising method at present.

4.1. Naked mRNA Injection

That is, mRNA is directly injected into the tissue without packaging, and the buffer is usually diluted before injection. Commonly used buffer solutions include sucrose solution, lactate Ringer's test solution, phosphate buffer solution, trehalose solution, etc. In 1990, Wolff et al. found for the first time that direct injection of mRNA in mice could also achieve normal expression of reporter genes in muscle cells, and the more mRNA injected, the higher the protein expression[2]. In 2000, Ingmar et al. directly injected naked mRNA into mice, and after 2 weeks, specific anti-β-glycosidase IgG could be detected in mice, and glycosidase-specific CTL response could also be triggered, indicating that injection of naked mRNA in mice could trigger humoral and cellular immunity specific to glycosidase[7]. In 2004, Carralot et al. also found that injecting naked mRNA encoding glycosidase in mice could detect normal expression of glycosidase in mice[8]. Regarding the uptake mechanism of naked mRNA, some studies believe that exogenous RNA is taken up by DC under the action of macrophages and obtained expression. Other studies have shown that the delivery efficiency of mRNA is affected by the type of buffer and the concentration of ions in the buffer. This delivery method has the advantages of low cost, convenience and fast, but due to the instability of mRNA and the complex physiological environment in the human body, RNA is easy to degrade and lose its role.

4.2. DC Load

DC, as the most powerful antigen presenting cell in the human body, plays a key role in the immune system and is considered to be a very promising strategy in anti-cancer immunotherapy. DC can load the mRNA encoding the desired antigen and cause cellular immunity. The study shows that electroporation is an efficient method to introduce mRNA into DC. Currently, an example of a DC-loaded mRNA vaccine is the Trimix DC-MEL vaccine given to melanoma patients. The vaccine is loaded with four mrnas, tumor antigens, CD40L, CD70 and active TLR4, and has been shown to be effective in clinical trials[9]. Although the efficiency of mRNA transfection with DC is low, and the long preparation time may lead to the loss of the MRNA-induced immune response, based on the important position of DC in the immune system, DC-loaded mRNA can still be an alternative direction for future immunotherapy research, especially the combination immunotherapy strategy of combining DC-based vaccines with other cancer therapies.

4.3. Cationic Peptide

Protamine is a naturally occurring protein that, due to its large amount of cationic L-arginine, can form complexes with negatively charged mRNA to protect nucleic acids from enzymatic degradation. An mRNA vaccine delivery system for rabies developed by CureVac using protamine has proven to be effective[10]. Even though the efficiency of protamine transfection mRNA needs to be improved, due to its clinical safety, it can still be used as a feasible research direction for mRNA delivery systems.

4.4. Cationic Nanoemulsion

Cationic nanoemulsion is an oil-in-water delivery method, usually consisting of squalene, indicating an active agent and a buffer, MF59 and AS03 are two examples of adjuvant systems, of which MF59 is permitted in many countries for pandemic and seasonal influenza vaccines due to its safety. AS03 has been shown to activate the immune system to increase antigen uptake and presentation, making it a promising direction for future research in this area[11,12]. The cationic nanoemulsion based on the adjuvant MF59 contains a key ingredient that combines mRNA in the oil phase to protect it from degradation. Gennova Biopharmaceuticals Ltd and HDT Biotech Corporation have jointly developed a lipid inorganic nanoparticle for the delivery of the SARS-Cov2 vaccine HGCO10, a material similar to cationic nanomilk. The superparamagnetic iron oxide (FE3O4) can achieve therapeutic and imaging functions, and the colloidal stability of this preparation is far better than that of the currently used SARS-Cov2mRNA vaccine[13].

4.5. LNP

Lipid nanoparticles(LNP) is the most advanced mRNA drug delivery method in the clinic, which has been demonstrated in the LNP-mrna vaccines Moderna and Pfe-Biontech in COVID-19. LNP includes cationic ionizable lipids, cholesterol, helper phospholipids, and polyethylene glycol lipids. The ionizable lipids envelope the negatively charged mRNA through a combination of electrostatic attraction and hydrophobic interaction. Cholesterol as a

neutral lipid can not only enhance particle stability, but also improve transfection efficiency. Polyethylene glycol lipids reduce antibody binding of serum proteins and clearance of phagocytes, thereby extending systemic circulation time. Auxiliary phospholipids can provide a bilayer stable structure, and it has also been demonstrated that the chemical properties of phosphoric acid play an important role in endosome escape[14]. In addition, there have also been reports of adverse events related to MRNA-LNP, inflammatory reactions in the body, and human tolerance to drugs[15], and further pharmacological and immunological studies on LNP components are needed to achieve safe and effective mRNA delivery. The in-depth research on mRNA-LNP has become a hot spot in the future treatment of cancer or infectious diseases.

5. saRNA

In addition to the traditional non-replicating mRNA, there is also a self-amplifying mRNA, which is based on the original mRNA, and there is an open reading frame at the five ends, encoding the four non-structural proteins (NSPs) of the specific virus. The structural protein virus gene behind the subgenomic promoter (SGP) was replaced with a heterogenic gene encoding the target protein. After the saRNA enters the host cytoplasm, the four non-structural proteins form an RNA-dependent RNA polymerase (RDRP) through a complex multi-step polymerization process that guides durable and efficient antigen expression. Due to its self-replicating properties, saRNA can be delivered at lower concentrations than conventional mRNA to achieve comparable antigen expression. ARCT-145 is the world's first commercially available saRNA vaccine. With the development of RNA technology, innovative changes related to saRNA technology are also emerging, such as taRNA and induced pluripotent stem cells. Despite some limitations, saRNA technology still has a wide range of application prospects and is expected to become a rising star in mRNA technology.

6. CircRNA

Another promising mRNA is circular mRNA, a single-stranded RNA that presents a covalent closed-loop structure and lacks the end sequence required for exonuclease recognition, so it is not easily degraded by exonuclease, is more stable than linear mRNA, and has advantages in delivery and treatment. Since it does not contain a cap and tail structure, it has advantages in terms of production. It has also been suggested that circRNA has a lower immune prototype, cytotoxicity, and can exhibit a more durable immune response. CircRNA have great potential to become the next generation of mRNA drug platforms, however, circRNA are still in the early stages in terms of design, synthesis, purification, delivery and application. More research and experiments are needed to prove its feasibility.

7. Conclusion and Prospect

With the deepening of the research on mRNA drugs, more possible research directions have been explored. A study has shown that a multivalent mRNA vaccine can express antigens of multiple known influenza virus subtypes, inducing the production of multiple antibodies. This makes it possible to develop a universal flu vaccine. Meanwhile, a multivalent mRNA vaccine against monkeypox virus showed a strong immune response. In addition, mRNA vaccines have emerged as promising cancer therapies. In addition to dealing with the possible emergence of infectious diseases, mRNA vaccines can also encode tumor antigens, enabling personalized therapies. An mRNA vaccine for pancreatic ductal cancer (PDAC) has been shown to be safe and feasible in a clinical trial, and further trials are being conducted [16]. There are also protein replacement therapies being explored, which translate proteins by transfecting mRNA into somatic cells to replace abnormal proteins or to supplement deficient proteins. mRNA can also be used in CAR-T cell therapy for autoimmune diseases, and a clinical trial in patients with myasthenia gravis demonstrated that this treatment is more effective and safer than DNa-based CAR-T therapy.

The key technologies in mRNA design include mRNA modification, in vitro transcription, purification, and delivery systems. At present, the application of these technologies has been basically mature, but there are still some challenges and obstacles to be faced. The first is the transportation and storage of mRNA vaccines. The storage conditions of most mRNA drugs are harsh, and the stability decreases rapidly with the rise of temperature. Many expired vaccines are wasted, especially in some resource-poor areas, and ultra-low temperature storage becomes a problem. Developing mRNA vaccines with high stability at relatively high temperatures or designing lower-cost mRNA kits is a possible but difficult strategy. In addition, because the clinical application of mRNA vaccines and drugs is relatively new, many safety issues or adverse events may occur, and how to effectively reduce the potential risks of mRNA drugs has become a key challenge.

In summary, mRNA drugs are a powerful and versatile platform. Due to its safety, rapid production capacity, flexibility, is considered to have great potential. But many challenges remain, and decades of research have laid a solid foundation for mRNA drug research, and further exploration is needed to understand their full potential. Going forward, it is recommended to focus research on delivery systems, especially LNP-based research. Also, keep an eye on possible security issues. In addition, combining mRNA drugs with other immunotherapies may be a good option. More clinical trials and data will enrich the understanding of mRNA drugs, which are becoming a sword in the treatment of infectious diseases and cancer.

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