Advances in Development of mRNA-Based Therapeutics

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Contents

Abstract Recently, mRNA-based therapeutics have been greatly boosted since the development of novel technologies of both mRNA synthesis and delivery system. Promising results were showed in both preclinical and clinical studies in the field of cancer vaccine, tumor immunotherapy, infectious disease prevention and protein replacement therapy. Recent advancements in clinical trials also encouraged scientists to attempt new applications of mRNA therapy such as gene editing and cell programming. These studies bring mRNA therapeutics closer to real-world application. Herein, we provide an overview of recent advances in mRNA-based therapeutics.

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1 Introduction

Great progress has been made in the development of nucleic acid-based therapeutic agents. Many drugs have already been approved for clinical applications by the regulatory bodies in the USA and in Europe. Gene therapy drugs Glybera (Watanabe et al. 2015), Strimvelis (Aiuti et al. 2017), Luxturna (Ginn et al. 2018) and Zolgensma (Rao et al. 2018), antisense oligonucleotide (ASO) drug Spinraza (Dolgin 2017), and small interfering ribonucleic acid (siRNA) drug Onpattro (Hoy 2018) are just a few examples of approved nucleic acid drugs. Messenger RNA (mRNA)-based therapeutics have a great potential in human applications. The mRNA molecule can be constructed to not only express conventional proteins for protein replacement therapy (Kormann et al. 2011) but also produce therapeutic antibodies (Kose et al. 2019). In addition, it can be tailored to encode chimeric proteins that are used for cell engineering and antigen proteins or peptides in vaccine development. Despite the great potential, clinical application of mRNA-based therapeutics has been lagged comparing to other types of nucleic acid drugs. One of the bottleneck issues has been difficulties in delivering mRNA molecules into the target organs and/or cells inside the body. Comparing to the ASOs and siRNA oligos, mRNA molecules are usually much bigger in size and are very sensitive to enzymatic degradation. Thus, technology platforms designed for ASO and siRNA cannot always be applied directly for mRNA delivery. Another technical challenge is synthesis of a large quantity of mRNA molecules for large-scale drug production. In this chapter, we will introduce recent progress in platform development and in vitro synthesis of mRNA molecules. In addition, we will review potential clinical applications of mRNA therapy, with primary focus on cancer immunotherapy and protein replacement therapy.

2 Delivery Platforms for mRNA Therapeutics

There has been a long history in the development of platforms for RNA transfection in vitro and RNA delivery in vivo. Verma and colleagues applied a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), to prepare liposomes and applied it to transfect luciferase mRNA into NIH3T3 cells back in 1989 (Malone et al. 1989). Hoerr and colleagues used protamine-condensed mRNA and liposome-coated protamine/mRNA complex to enhance the stability of mRNA and uptake of mRNA into cells in vivo in 2000 (Hoerr et al. 2000). Multiple lipid-based platforms have been described since then. Among the variety of technology platforms, lipid nanoparticle (LNP) is one of the most widely used

formulations for in vivo applications (Semple et al. 2010; Genc et al. 2011; Owen et al. 2018). Although initially designed for delivery of small molecule drugs, LNPs have been adapted to deliver siRNA oligos (Akinc et al. 2010), plasmid DNAs (Vijayanathan et al. 2002), and most recently mRNAs (Thess et al. 2015; Guimaraes et al. 2019). LNPs are usually less than 100 nm in diameter and are composed of structural phospholipids, cholesterol, and cationic lipids. DlinMC3DMA is one of the cationic lipids that have been successfully applied in mRNA delivery (Yanez Arteta et al. 2018). Many laboratories and companies have developed various forms of LNP-based platforms for mRNA delivery. Rosigkeit, S and colleagues developed a LPX delivery platform that was composed of DOTMA and dioleoyl phophotidylethanolamine (DOPE) and applied it to deliver mRNA and induce immune responses by targeting lung or spleen through the adjustable surface potential of nanoparticles (Rosigkeit et al. 2018). Kowalski, P. S. and colleagues applied liposome consisting of lipid and lipid-like polymer to deliver mRNA for protein replacement therapy (Kowalski et al. 2018). Patel, S. and colleagues applied LNP with novel lipid structures to increase protein expression efficiency (Patel et al. 2017; Hassett et al. 2019). Since most lipids can be dissolved in ethanol, the lipids can be mixed with nucleic acids in water in a microfluidic setting (Belliveau et al. 2012). This allows for controllable and standardized large-scale production. Indeed, precision nanosystems (www.precisionnanosysytems.com) have developed microfluidics-based instruments to prepare LNPs at different scales including one for GMP-grade drug manufacturing.

Other delivery platforms have also been optimized to achieve maximum delivery efficiency and protein expression. They include lipoplex (Koynova et al. 2007), cationic peptide (Yang et al. 2009), cationic polymer (Green et al. 2008), and micelle (Zheng et al. 2013). Persano, S. and colleagues applied a lipopolyplex (LPP) delivery platform which is composed of a polymer-condensed mRNA inner core surrounded by a lipid shell (Persano et al. 2017). Preliminary results suggest effective delivery of mRNA molecules to dendritic cells and potent protein expression.

Despite the tremendous advances in the field, there is still a big demand for new delivery platforms that are applicable for different routes of administration routes such as intravenous, intradermal, subcutaneous, intramuscular, and intratumor injections (Pardi et al. 2015). These platforms are expected to provide an optimal biodistribution pattern and to enable effective endosomal escape of mRNA molecules so as to improve protein expression. In addition, new platforms should also have an ideal biocompatibility.

3 In Vitro Synthesis of mRNA Molecules

Production of a large quantity of mRNA molecules is vital for the success of development of mRNA therapeutics. Although there are a few commercial entities that sell mRNA molecules, the list of mRNAs on their catalogs is not long. Thus, most laboratories and biopharmaceutic companies rely on their own facility or contract research organizations (CROs) to produce mRNAs, most through in vitro

transcription (IVT). Although the technology has been improved dramatically in the last three decades, bacteriophage-derived RNA polymerases have served as key enzymes for in vitro mRNA synthesis throughout the time (Sarnow 1989). A single strand DNA that contains a 5′ untranslated region (UTR) including a T7 promoter, an open-reading frame of the gene-of-interest, and a 3′ polyA tail is used as the template in the IVT. An anti-reverse di-guanosine cap analog is included in the IVT to generate a 5′ cap (Warren et al. 2010). However, it was found that the synthetic mRNA molecules may cause undesirable immune responses (Linares-Fernández et al. 2020). High performance liquid chromatography (HPLC) has been applied to purify the IVT product so as to mitigate the side effects (Kariko et al. 2011). In addition, pseudouridine and other modified nucleosides have been incorporated into the synthesized mRNA molecules to suppress immune responses, to increase translational efficiency, and to enhance mRNA stability (Warren et al. 2010; Kariko et al. 2008).

4 Clinical Application of mRNA Therapeutics

4.1 mRNA in Cancer Immunotherapy

Cancer immunotherapy has gained much attention in recent years due to its huge success in patient care with therapeutic antibodies, T cell-based therapies, and cancer vaccines (Mellman et al. 2011). Although no approved mRNA drug is available for now, mRNA-based therapy has played a big role in the field of cancer immunotherapy (Foster et al. 2019; Diken et al. 2017). Owing to their versatile applicability, mRNA molecules have been used to generate tumor-associated antigens and neoantigens in cancer vaccine development (Tanyi et al. 2018), in therapeutic T cell manipulation (Beatty et al. 2014), in antibody production (Stadler et al. 2017), and in expression of therapeutic cytokines (Hewitt et al. 2019). Comparing to proteins and peptides, mRNA may offer unprecedented advantages, such as generating diversified tumor antigens from one single molecule as a result of alternative mRNA splicing or intron retention (Frankiw et al. 2019; Smart et al. 2018). In Sects. 4.2–4.4, we will describe mRNA-based cancer immunotherapeutics in detail. In the meantime, mRNA vaccines have also been successfully applied in the fight against infectious diseases, such as the Zika virus (Richner et al. 2017a, b). Details will be described in Sect. 4.5.

4.2 Cancer Vaccine

Applying mRNA encoding tumor antigens has unique advantages over the traditional protein or peptide-based vaccine strategies. mRNA molecules serve as self-adjuvants (Ziegler et al. 2017). There is no limitation on human leukocyte antigen (HLA)-type restrictions. Comparing to DNA-based therapies, mRNA

vaccines do not integrate into the genome and therefore do not generate the risk of gene mutation. In addition, mRNA vaccines are adaptable to both dividing and non-dividing cells. Mechanistically, mRNAs encoding cancer antigens are delivered to the antigen-presenting cells (APCs) where they are translated in the cytoplasm. The newly synthesized protein is then processed into peptides, and the generated antigen peptides are presented by major histocompatibility complex (MHC) class I or MHC class II molecules which then activate T cells (Fiedler et al. 2016). Both MHC-TCR and B7-CD28 interactions are needed to generate antigen-specific T cells and to promote T cell proliferation. In order to develop a potent therapeutic cancer vaccine, it is essential to select the proper tumor antigen and adjuvant(s).

4.2.1 Tumor-Associated Antigen (TAA)-Based Cancer Vaccine

TAAs are antigens that are overexpressed in tumor cells. They are the primary choice for cancer vaccine development. In some of the pilot studies, scientists in CureVac demonstrated antigen generation from intradermally injected naked mRNA, cationic liposomal mRNA or protamine complex-encapsulated mRNA, and these mRNA vaccines induced both antigen-specific cytotoxic T lymphocytes (CTLs) and IgG antibodies (Hoerr et al. 2000). In addition, they found that T helper 2 (Th2)-type immune responses could be induced by intradermal vaccination of naked β-globin untranslated region (UTR)-stabilized mRNA, and that Th2-biased response could be shifted to a Th1-type response by co-delivering granulocyte–macrophage colony stimulating factor (GM-CSF) (Carralot et al. 2004). Early clinical trials demonstrated that intradermal injection of protamine-complexed mRNA or mRNA combined with GM-CSF was feasible, safe and effective, and treatment successfully induced antigen-specific T cell and antibody immune responses (Rittig et al. 2011; Weide et al. 2008). The strategy was further optimized by packaging two mRNA components in one formulation: a naked mRNA to encode the tumor antigen and a protamine-complexed mRNA to stimulate Toll-like receptor 7 signaling. The two-component mRNA vaccines with self-adjuvant property induce balanced adaptive immune responses and significantly better antitumor activity compared to single-component mRNA vaccine (Fotin-Mleczek et al. 2011). Apart from that, it is important to note that the strategy of using mRNA encoding a single antigen may not have enough immunogenicity to break central immune tolerance (Vansteenkiste et al. 2016). Therefore, strategies based on antigen cocktail were applied to maximize the immunogenicity of vaccines. Recent clinical results showed that CV9201, a RNA cancer vaccine encoding five non-small cell lung cancer antigens (NSCLC) (Sebastian et al. 2019), exhibited a well-tolerated safety profile and significantly improved immune responses against TAAs in patients who received a dosage of 1600 μg (NCT00923312). In another study, they combined BI1361849 (CV9202), a self-adjuvanted vaccine formulation consisting of protamine-complexed mRNA encoding six antigens (Papachristofilou et al. 2019), with local radiotherapy to reverse the immunosuppressive tumor microenvironment through induction of immunogenic tumor cell death and to

enhance recruitment and stimulation of T cells in patients with stage IV NSCLC. Results from a phase Ib trial showed that treatment was well tolerated and induced antigen-specific response in 84% patients. In addition, 46.2% patients achieved stable disease after vaccination (NCT01915524). Current clinic trial is ongoing to evaluate the effect from CV9201 in combination with the checkpoint blockade antibody durvalumab (NCT03164772).

4.2.2 Neoantigens Antigen-Based Cancer Vaccine

Neoantigens are generated when mutations are introduced in cancer cells (Schumacher and Schreiber 2015). Compared with non-mutated self-antigens, neoantigens may contribute more significantly to tumor control, since T cells stimulated by neoantigens tend to avoid central immune tolerance (Gilboa 1999). In order to identify neoantigens, DNA samples from both tumor and normal tissues are sequenced, and results are used to predict binding affinity from mutant proteins to patient's HLA alleles. Mutant antigen peptides are then ranked, and the information is applied to synthesize peptides or neoantigen-encoding mRNAs that will be used for vaccine preparation (Grabbe et al. 2016). DNA sequencing technology has been advanced so dramatically in recent years, and machine learning algorithms are being used to predict mutated peptides binding with HLA molecules (Linnemann et al. 2015; Abelin et al. 2017; Fritsch et al. 2014; Bulik-Sullivan et al. 2018). Applying mass spectrometry has also greatly advanced the field of tumor antigen identification (Creech et al. 2018). Consequently, personalized vaccines for cancer immunotherapy have been remarkably promoted. Preclinical studies from BioNTech AG revealed that one-third of mutated epitopes identified from B16F10 murine melanoma were immunogenic (Castle et al. 2012). Intravenous administration of neoantigen-encoding mRNA in lipoplex induced interferon-α secretion by plasmacytoid DCs and macrophages and promoted strong immune responses including maturation of DCs, and proliferation of antigen-specific effector and memory T cell. The immune responses subsequently mediated potent IFNα-dependent rejection of progressive tumors (Kranz et al. 2016). There have also been reports that showed an important role from CD4+ T cells in remodeling the tumor microenvironment after MHC class II-restricted epitopes administration (Sebastian et al. 2015). Proof-of-concept personalized cancer immunotherapy with mRNA cancer vaccine was first demonstrated in 2017 in patients with melanoma (Sahin et al. 2017). T cell immune responses to multiple neo-epitopes were induced in the patients who received treatment with mRNA cancer vaccines. Neoantigens-specific T cell responses were detected after vaccination in two patients with resected metastases, and one of them achieved a complete response after treatment with vaccine in combination with PD-1 inhibition therapy (Vallazza et al. 2015). Multiple clinical trials using mRNA-based cancer vaccines are currently being conducted in multiple cancer types, such as melanoma, colorectal cancer, glioblastoma, non-small-cell lung cancer, esophageal cancer, and bladder cancer (NCT03480152) (Li et al. 2014).

4.2.3 Dendritic Cell Vaccine

Since DCs are professional APCs, they can be loaded with tumor antigens to induce anti-cancer immune responses. mRNA in DCs can serve both as the source for antigen production and a potent adjuvant to stimulate TLR7/8 signaling (Laurent et al. 2012). First DC vaccine pulsed with mRNA was reported in 1996, and results showed that exposure of DCs to antigen-encoding mRNA or total mRNA extracted from tumor cells could induce significant T cell immune responses and inhibit growth of established tumors (Boczkowski et al. 1996). Many clinical trials using mRNA-based DC vaccines have been performed in cancer patients since then, and feasibility and safety of this treatment strategy have been well established (Gerold 2010; Daphné et al. 2015; Krug et al. 2014). In addition, efficacy from mRNA-transfected DCs can be further enhanced by combining cytokines and checkpoint blockade inhibitors in treatment (Mu et al. 2005; Kyte et al. 2006). It is important to point out that the cytokines and antibodies in combination treatment can also be produced by mRNA molecules inside the cells. DCs displayed a strong stimulatory potential after transfection with mRNAs encoding IL-12, IL-18 or other proinflammatory cytokines (Bontkes et al. 2007; Bontkes et al. 2008). Introduction of mRNA encoding the soluble extracellular part of PD-1 or PD-L1 resulted in elevated levels of CD80 (a DC maturation marker) and a group of cytokines and induced multifunctional T cells and cytokines secretion (Pen et al. 2014). Since DCs activation is mediated by several pathways, combination treatment of DCs to stimulate these pathways may achieve even better outcomes. TriMix-DC vaccine, a DC vaccine with mRNA molecules that encode TLR4, CD40L and CD70 (Pen et al. 2013), showed superior stimulatory capacity and suppressed the activity of regulator T cells (Treg), thus lifting CD8+ T cell activity.

4.3 Therapeutic mRNA Encoding Cytokines and Other Immune-Modulating Factors

mRNAs encoding therapeutic cytokines, checkpoint blockade antibodies, and immune agonists have the potential to convert an otherwise non-inflamed tumor (that lacks T cell infiltration, also known as immunologically "cold") into an inflamed tumors (Galon and Bruni 2019). This group of reagents is under extensive investigation in both preclinical and clinical studies. Hewitt and colleagues designed a triple combination therapy for intratumor injection of mRNAs encoding IL-36 γ, IL-23, OX-40L to turn "cold" tumors into "hot" tumors. Animal studies showed that mRNA mainly expressed in tumor tissues and triple therapy successfully modified the tumor microenvironment. The reagents stimulated both the innate and adaptive immune system by stimulating production of cytokines including IL-6, IL-22, TNF-α, IFN-γ, and IL-1β, promoting proliferation and infiltration of immune cells (DCs, NK cells, CD4+/CD8+ T cells) in both tumor

tissue and proximal lymph nodes without affecting Treg cells. As a result, treatment of MC-38 colorectal tumor-bearing mice with cytokine-encoding mRNA via intratumoral injection dramatically inhibited tumor growth (Hewitt et al. 2019). The result showed that intratumoral triplet mRNA therapy may avoid systemic toxicity and drive in vivo immune activation against tumor antigens and obtain a long-term therapeutic effect. Clinic trials have been initiated to evaluate potential toxicity from mRNA encoding IL-12, OX 40L monotherapy (NCT03323398) and mRNA encoding IL-36 γ, IL-23, OX-40L triple therapy (NCT03739931). Intratumoral delivery of mRNA has also been applied to produce other cytokines and chemokines including a fusion protein composed of interferon-β and the extracellular binding domain of the TGF-β receptor II (Van der Jeught et al. 2014). In addition, the strategy has been used to produce antibodies against cytokines IL-6 and TGF-β (Bialkowski et al. 2018). Furthermore, intratumoral delivery of mRNA has been applied to produce recombinant bacteriophage MS2 virus-like particles (VLPs) (Harper and Sardh 2014), mAbs targeting checkpoint molecules (PD-1, TIM-3, LAG-3), and necroptosis executioner mixed lineage kinase domain-like (MLKL) protein (Van Hoecke et al. 2018).

4.4 CAR-T Cells

Clinical trials showed that engineering T cells with chimeric antigen receptors (CARs) or T cell receptors (TCRs) have a significant therapeutic benefit on patients with relapsed or refractory hematological malignancies (Cummins and Gill 2018). The first CAR-T (Kymriah) product was approved in 2017 for treatment of patients with acute lymphoblastic leukemia. Transfection of T cells with mRNA to express CAR has the potential to temporally limit the targeting capacity of genetically modified T cells due to transient CAR expression and therefore to reduce the potential of sustained killing of normal cells that express the targeted TAAs such as mesothelin (Hung et al. 2018), EGFR (Caruso et al. 2016), CD19 (Caruso et al. 2016), CD20 (Panjwani et al. 2016), CD33 (Kenderian et al. 2015). Expression of CAR from introduced mRNA has been shown to transiently redirect T cell specificity to a desired TAA and mediate tumor regression in murine models of mesothelioma and leukemia (Yangbing et al. 2010; Barrett et al. 2013). A new approach of engineering T cells using IVT mRNA to transiently express CAR including both the CD3-ξ and 4-1BB for reducing off-target toxicity is under clinical evaluation. Clinical results indicated CAR-Ts generated from adoptively transferred mRNA to target mesothelin are feasible and safe (Beatty et al. 2014). The lifespan of CART-meso cells was short in the peripheral blood after intravenous injection, and these CAR-T cells effectively migrated to primary and metastatic tumor sites. Bai and colleagues reported an approach that used modified mRNA encoding telomerase reverse transcriptase to transfect CD19 CAR-T cells in order to improve their lifespan and proliferation (Bai et al. 2015). mRNA treatment instantly boosted telomerase activity in the new CD19 CAR-T cells, which

promoted proliferation, delayed replicative senescence, and then, provided long-term antitumor activity in a mouse xenograft model of B-cell leukemia (Bai et al. 2015).

4.5 mRNA Vaccines in Infectious Diseases

mRNA vaccines have been applied for prevention of infectious diseases such as influenza viruses (Richner et al. 2017; Petsch et al. 2012), zika virus (Feldman et al. 2019), rabies virus(Schnee et al. 2016), and Dengue virus (Roth et al. 2019). In 2017, a research group published a study on mRNA vaccines to protect against Zika virus infection (Richner et al. 2017). In these vaccines, the mRNA molecules encoding Zika viral prM/M-E protein antigens contain a proprietary nucleoside modification to minimize indiscriminate activation of innate immunity, although detailed information on the modification was not provided. In addition, a donor methyl group S-adenosylmethionine was added to the methylated capped RNA to enhance translation efficiency. The mRNA molecules were then packaged into lipid nanoparticles before the vaccines were applied to treat mice. The researchers found that a modified mRNA vaccine could prevent Zika disease in animal models. In a follow-up study, the researchers demonstrated protection against Zika virus-induced congenital disease in mice (Richner et al. 2017). With the recent emergence of the corona virus, there is an international effort to develop both prophylactic vaccines and therapeutic vaccines (Steenhuysen and Kelland 2020). Moderna and CureVac announced their involvement in company news release, and Stemirna has applied the LPP platform in vaccine development. Exciting development is anticipated in the coming months. For a more detailed overview of this mRNA application, please refer to the review in the book chapter "Messenger RNA-based vaccines against infectious diseases" reviewed by Mohamad-Gabriel Alameh, Drew Weissman, and Norbert Pardi.

4.6 mRNA in Protein Replacement Therapy

Defective protein translation from DNA genetic information can give rise to various diseases, such as fabry disease, methylmalonic acidemia (MMA), acute intermittent porphyria (AIP), Hemophilia B, and cystic fibrosis (CF) (Kerem et al. 1989; Mehta et al. 2004; Lerner-Ellis et al. 2006; Koeberl et al. 1990). In addition, normal proteins may not be available in certain disease areas due to blood vessel damage, leading to further development of diseases such as heart failure and diabetes foot. Compared with conventional protein drugs for such indications, mRNA therapy provides an effective alternative, since a single mRNA molecule can be translated into a large quantity of protein molecules over the course of hours or days treatment time (Warner et al. 1963). Furthermore, the nascent protein will go through all the

required post-translational modification procedures including phosphorylation, acetylation and glycosylation, a fully functional protein product is guaranteed (Helenius et al. 2013).

4.6.1 Local Injection-Based Protein Replacement Therapy

Myocardial Infarction

Despite advances in curative and preventive medicine, cardiovascular disease still remains one of the leading causes of morbidity and mortality worldwide (Savarese and Lund 2017). To date, a significant clinically feasible and/or verified targeting biologic strategy for treating congestive heart failure is still lacking. Vascular endothelial growth factor A (VEGF-A) has been previously reported to regulate new blood vessel formation, enhance endothelial proliferation from epicardial-derived progenitor cells, and with a pro-survival effect on vascular, endothelial, and cardiac cells (Ferrara et al. 2003; Lui et al. 2013). Same as mRNA molecules for cancer therapy, those used for protein replacement therapy need to overcome the same technical hurdles as immunogenicity, instability, and low expression efficiency, and nucleoside modifications and 5' capping have been applied in mRNA production (Kariko et al. 2008; Mockey et al. 2006). The modified RNA synthesized by Zangi and colleagues showed high production efficiency and dose-dependent expression profile of VEGF-A in a murine myocardial infarction model (Zangi et al. 2013). Direct in vivo comparison of mRNA therapy and DNA therapy showed that the rapid, pulse-like expression profile of mRNA benefited growth of functional vessels, whereas the prolonged VEGF-A expression profile of DNA-induced toxicity due to redundant formation of leaky vessels (Zangi et al. 2013). An effect of expansion and directed differentiation of endogenous heart progenitor cells were shown from the intramyocardial injection of mRNA encoding VEGF-A in the murine myocardial infarction model. Furthermore, Leif and colleagues investigated potential therapeutic application of mRNA in ischemic heart disease in swine (Leif et al. 2018). The purified and optimized (optimization of nucleotide, UTR sequence, capping efficiency, and buffer solution) mRNA showed tissue specific and long-lasting expression of protein without triggering innate immune response. Moreover, the study showed that swine cardiac function was improved after a single intra-cardiac injection of VEGF mRNA one week after myocardial infarction through limiting cardiac infarct expansion and fibrosis, improving systolic function.

Diabetes Foot

Diabetic ischemic ulcer is an intractable and the most devastating diabetic complication. Similar to cardiovascular diseases, angiogenesis is a critical factor for diabetic wound healing (Varu et al. 2010; Harold and Marjana 2007). Application

of VEGF-A and PDGF-β offers a promising approach for treatment of wound and ulceration (Shi et al. 2018). Sun and colleagues analyzed microvascular responses after mice were treated with mRNA therapy (mRNA encoding VEGF-A, AZD8601) or protein therapy (VEGF-A protein) (Sun et al. 2018). Intradermal injection of AZD8601 into mice resulted in dose-dependent vasodilation, upregulation of blood flow and formation of neo-vessel in injection site, results that were not observed in mice treated with the VEGF-A protein or phosphate buffer saline control. In addition, sequential dosing of AZD8601 in diabetic mice resulted in sustained vascularization and tissue oxygenation within wound area. A clinical trial aiming to assess safety and potential therapeutic effects of the mRNA encoding VEGF-A on treating type 2 diabetes mellitus (T2DM) was performed (NCT02935712) (Gan et al. 2019). It was revealed that intradermal VEGF-A mRNA injection was well tolerated and local functional VEGF-A protein was steadily expressed after administration, which led to transient skin blood flow enhancement in patients with T2DM.

4.6.2 Liver Diseases

Liver is the natural target for protein replacement therapy, since most nanoparticles tend to accumulate in this organ via intravenous injection (Fenton et al. 2016; Derosa et al. 2016). Fabry disease is one of these diseases that can be effectively treated with protein replacement therapy. This is a rare inherited disorder of glycosphigolipid metabolism caused by absence or markedly deficient activity in liver α-galactosidase A (α-Gal A), an enzyme that is normally produced by the liver. Patients suffer from progressive decline in renal and cardiac function and develop cardiomyopathy and end-stage renal disease (Hebert et al. 2013; Messalli et al. 2012). mRNA molecules encoding α-Gal A were packaged by two different research groups into LNPs (Zhu et al. 2019) or nanoparticles formulated with lipids and lipid-like materials (DeRosa et al. 2019). A single intravenous injection of α-Gal A mRNA caused not only dose-dependent protein expression and substrate reduction but also long-term (up to 6 weeks) substrate reductions in tissue and plasma in mice. In addition, the product proved to be safe after multiple administrations to non-human primates (Zhu et al. 2019). Hemophilia B is another liver disease that is caused by a deficiency of factor IX (FIX), a serine protease. FIX activation plays a major role in the signaling the coagulation cascade (Jiang et al. 2018). The disease is characterized as sustained, internal bleeding, and easy bruising. The prophylactic treatment is an intravenous application of purified FIX along with blood transfusion, but a heavy administrative burden with continuous treatment over a short period was needed to ensure adequate as-needed dosage given. Ramaswamy and colleagues applied lipid-enabled and unlocked nucleic acid modified RNA (LUNAR) to treat FIX-deficiency and demonstrated its feasibility in mouse model of FIX-deficiency (Ramaswamy et al. 2017). LUNAR is a unique LNP composed of four lipids including a proprietary lipid. Delivery of human FIX mRNA encapsulated in LUNAR resulted in rapid pulse of the FIX protein, and high

protein concentration was maintained for 4–6 days. Therapeutic efficacy from mRNA therapy was comparable to recombinant human FIX protein therapy which is the current standard of care (Ramaswamy et al. 2017). An additional example of protein replaces therapy with mRNA for liver diseases include deficiency of methylmalonyl-CoA mutase (MUT), a vitamin B12-dependent mitochondrial enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA (Chalmers and Lawson 1982; An et al. 2017).

4.6.3 Lung Diseases

Lung is another organ that offers easy access to drug nanoparticles. Non-invasive aerosol inhalation is a defined method for delivering drugs to the lung, and many technology platforms have been developed for this purpose. For an example, Patel and colleagues synthesized hyperbranched poly (beta amino esters) (hPBAEs) to prepare stable and concentrated mRNA polyplexes for inhalation (Patel et al. 2019). They achieved 24.6% transfection efficiency in lung epithelial cells after a single dose. Cystic fibrosis which is caused by mutations in the CFTR gene is the most widespread life-limiting autosomal-recessive disease in Caucasian (Cutting 2015). Robinson and colleagues applied LNP-packaged mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) to treat cystic fibrosis (Robinson et al. 2018). Mutations in the CFTR gene cause abnormal flux of ions into and out of cells, leading to accumulation of thick airway mucosa and permanent tissue scarring and respiratory failure (Welsh 1990; Lyczak et al. 2002). Nasal application of LNP-CFTR mRNA in CFTR knockout mice recovered CFTR-mediated chloride secretion to conductive airway epithelia for at least 14 days and achieved comparable outcomes with currently approved drug ivacaftor (Robinson et al. 2018).

4.7 Genome Editing

Many genome editing tools have emerged in the past decade including zinc finger nucleases (ZFNs) (Miller et al. 2007), transcription-activator like effector nucleases (TALENs) (Wefers et al. 2013), and the clustered regularly interspersed palindromic repeats (CRISPR)/CRISPR-associated (Cas) enzyme system (Ran et al. 2013). However, off-target effects remain as a major concern. Since the nuclease activity is required for only a short period of time for the action, transient expression of mRNAs encoding ZFNs, LENs, and Cas9 provides a valid option to reduce off-target effects. Recently, Conway and colleagues showed that LNP-packaged mRNA encoding ZFNs to target the TTR and PCSK9 genes achieved over 90% knockout in gene expression in mice (Conway et al. 2019). Finn and colleagues applied LNP to load with Cas9 mRNA and sgRNA delivery system in order to edit the mouse transthyretin (Ttr) gene in the liver and achieved over 97% reduction of the protein level in serum, and the effect lasted for over

12 months after a single administration (Finn et al. 2018). Meanwhile, Miller and colleagues developed zwitterionic amino lipid (ZAL)-based delivery system to co-deliver Cas9 mRNA and sgRNAs and observed permanent DNA editing with 95% decrease in protein expression (Miller et al. 2017). These results suggest that mRNAs packaged in nanoparticles provide an excellent route for genome editing.

5 Conclusions

Application of novel therapies and medical technologies has revolutionized patient care. With the advance in mRNA synthesis and improvements in delivery platforms, mRNA-based therapies will play more and more important roles in the field. As with other types of therapeutic agents, the pharmaceutic industry has taken a very prominent role in advancing mRNA therapies. They have pioneered in mRNA modification in order to minimize innate immune responses. In the meantime, there is a constant need to understand the physical and biological barriers in mRNA delivery and to develop next-generation platforms so as to better overcome the barriers and achieve precision tissue- and cell-targeted delivery, beyond the liver and lung. In addition, approaches to enhance stability of mRNA molecules should continue to be explored. Furthermore, there is a need to standardize production of mRNA molecules to ensure high quality and efficacy of mRNA therapies. Finally, advances in machine learning and bioinformatic analysis will further facilitate sequence optimization of mRNA and medical application of therapeutic mRNA.

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