Molecular Therapy
Review

Delivery of mRNA Therapeutics for the Treatment of Hepatic Diseases

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Promising improvements in the field of transcript therapeutics have clearly enhanced the potential of mRNA as a new pillar for protein replacement therapies. Synthetic mRNAs are engineered to replace mutated mRNAs and to be immunologically inconspicuous and highly stable while maximizing protein expression. Approaches to deliver mRNA into the cellular cytoplasm safely and efficiently have been further developed so that two mRNA-based approaches replacing vascular endothelial growth factor (VEGF) and cystic fibrosis transmembrane conductance regulator (CFTR) have now made it into clinical trials. These studies bring mRNA therapeutics for protein replacement therapy closer to clinical realization. Herein, we provide an overview of preclinical and clinical developments of mRNA therapeutics for liver diseases.

The general concept of conventional gene therapy is the delivery of DNA or RNA encoding for a defective or missing protein(s) into the patient's cells. There, it serves as a template for protein synthesis and enables the cell to produce the missing or defective protein(s) on its own. This is advantageous over therapies directly applying the missing or defective protein, as they are relatively faster and cheaper to develop[1] without extensive size constraints. Moreover, certain proteins, e.g., membrane proteins or intra-organelle proteins, may be either difficult or impossible to produce *in vitro*. Besides production issues, correct post-translational modification(s) of therapeutic proteins (as would be the case when the protein is produced by the target cells) cannot be ensured after *in vitro* protein production in heterologous systems.[2,3]

Even though mRNA was discovered in 1961, the first successful demonstration of mRNA delivery to cells using liposomes was provided by Malone et al. in 1989.[4–7] In 1990, Wolff et al.[8] showed, for the first time, the therapeutic potential of mRNA, although at that time mRNA molecules were perceived as being impractical for therapeutic applications due to their instability. Besides instability, another feature associated with *in vitro*-transcribed mRNA is the immunogenicity of the molecule. Incorporation of modified nucleotides into the mRNA molecule significantly improved its translation and half-life and resulted in a significant reduction in its immunogenicity.[9–11]

Since then, mRNA has moved from being a fragile molecule in research laboratories into clinical studies. The evolutionary development of mRNA therapeutics has been well captured in recent reviews.[2,3,12,13] The initial wave of mRNA therapeutics has focused on its use in cancer immunotherapy involving an *ex vivo* transfer of vaccinations and localized *in vivo* delivery.[14–16] The most advanced candidates of mRNA therapeutics are currently in phase II clinical testing.[17] In contrast to vaccination and cancer immunotherapies, the majority of protein replacement therapies using mRNA are still in preclinical development. A couple of molecules that have made it to clinical trials are mRNAs encoding for vascular endothelial growth factor (VEGF) (phase II: AstraZeneca and Moderna) and cystic fibrosis transmembrane conductance regulator (CFTR) (phase I: TranslateBio). For an overview of all therapeutic mRNA applications, please refer to the review from Hajj and Whitehead.[12]

DNA versus RNA

DNA-based approaches have been proposed to be more suitable for the treatment of hereditary diseases caused by missing or dysfunctional proteins. However, in the case of DNA therapeutics, not only high cytoplasmic DNA delivery but also efficient nuclear entry of DNA cargo has to be ensured, as the DNA needs to get transcribed into mRNA before the therapeutic protein molecule can be produced. As the majority of the terminally differentiated cells are post-mitotic cells, which do not undergo frequent cell division *in vivo*, nuclear delivery is a major hurdle for DNA therapeutics.

When comparing DNA and mRNA therapeutics, there are several advantages to using mRNA: it does not need to enter the nucleus; it cannot integrate into the genome; and the degradation of mRNA can be determined through its structure, thereby allowing better controllable pharmacokinetics of the drug.[13] Based on therapeutic needs, the transient nature of mRNA also turns out to be the limitation of transcript therapies for genetic and metabolic diseases where lifelong treatment (and repeated dosing) with the therapeutic molecule is required. Irrespective of the treatment regimen (transient

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versus lifelong), the major objective of mRNA therapy is to successfully deliver the therapeutic mRNA into the cytoplasm, resulting in efficient translation of the molecule with negligible toxicity and immunogenicity. These essential prerequisites have to be fulfilled in order to enable a sufficiently high level of encoded protein leading to a therapeutic benefit.

The Concept of mRNA Therapy

To achieve the purpose of mRNA-based therapy, i.e., enabling the cell to produce the missing and/or defective protein, the therapeutic mRNA molecule needs to enter the patient's cells and has to be translated in the cytoplasm efficiently enough to result in therapeutic levels of the missing and/or defective protein. For this, the mRNA molecule needs to overcome several hurdles on its way to the ribosomes in the cytoplasm.[3,12] One major hurdle is the fragility of mRNA owing to its chemical composition. The hydroxylation of the ribose 2′ position makes the molecule highly susceptible to hydrolysis and pH shifts. The incorporation of uridine instead of thymidine nucleosides, its length (100 to several thousand nucleotides), and its complex secondary structure bring in additional complexity compared to DNA and other RNA molecules (e.g., small interfering RNA [siRNA], antisense oligonucleotides, etc.). These chemical and structural parameters need to be kept in mind when designing suitable mRNA delivery systems, and they affect both mRNA stability and uptake.[18,19] However, knowledge gained through extensive research activities in the last two decades enables one to optimize the mRNA molecule in order to maximize transport, stability, efficiency, and, thus, the therapeutic potential. Based on the requirements, the following structural components of therapeutic mRNA molecule can be modified:

5′cap: it is required for mRNA processing, nuclear export, and translation initiation.[20] Various cap analogs were synthesized and tested for their ability to confer resistance to enzymatic cap cleavage. Anti-reverse cap analogs (ARCAs) are the most useful modifications. There are different ARCA variations; one is the β-S-ARCA, which is resistant to decapping by DCP2. For more detailed information, readers can refer to previously published works.[21–24]

Poly(A)tail: it regulates stability and translational efficiency of the mRNA molecule. In dendritic cells, it was shown that the optimal length is between 120 and 150 nt.[3,25,26]

5′ and 3′ UTRs: UTRs can be modulated or exchanged in order to increase translation and stability. For example, the 3′ UTR of human α- and β-globin enhances stability and translation of mRNAs. It was also shown that the 3′ UTR of human β-globin arranged in a head-to-tail orientation additionally increases mRNA stability.[3,27,28]

Coding sequence: the coding sequence also influences the translational efficiency, since it is known that there exist preferred codons that lead to an optimized and accelerated translation compared to rare codons.[29] Moreover, nucleotide modification is another tool to improve mRNA translation by improving its stability and reducing its immunogenicity.[9,27,30,31] Recently, Thess et al.[32] demonstrated the potential of mRNA sequence optimization, as they reported optimized sequences that were non-immunogenic and resulted in higher protein when used as unmodified mRNA compared to their base-modified counterpart. On the contrary, the incorporation of modified bases in those optimized sequences reduced the resulting protein yield.[32]

Furthermore, ubiquitously present blood and tissue RNases are a significant hindrance to the systemic applicability of naked mRNA.[33] At the cell surface, the next barrier that needs to be overcome is the negatively charged lipid bilayer. Molecules smaller than 1,000 Da can passively diffuse across the membrane, while naked RNA is a large and negatively charged molecule that is hardly transported into the cell without a carrier.[19] Some cell types can spontaneously uptake naked RNA, however, the rate is less than 1 in 10,000.[3] Delivery systems transport the mRNA in nanometer-sized particles (<1 μm) and enable cellular entry, reversible condensation of mRNA, and localization in the cytoplasm by overcoming acidic endolysosomes.[34–36] The mechanism by which RNA carrier particles enter the cell depend on their size: particles smaller than 200 nm are taken up by clathrin-mediated endocytosis, whereas the entry of bigger particles occurs in a clathrin-independent manner.[37]

Once an RNA molecule has made it into the cell, the final challenge is to get released (without being degraded) from the endosome into the cytoplasm. Previous reports estimate that only about 2% of the RNA is released from the endosomes, and Lorenz et al.[18] have shown that naked mRNA that entered the cell via receptor-mediated endocytosis remains in the endosomes. This shows that the process of endosomal escape is a limiting factor of delivery and supports the thesis that cellular entry alone is not sufficient to ensure the translation of mRNA.[12,38,39] Instead, it is the efficient release from the endosomes and cytoplasmic localization of the mRNA that need to be ensured in order to achieve translation of the molecule.[18]

On the way of the mRNA to the ribosomes, further obstacles are evolutionarily conserved defense mechanisms, like the innate immune Toll-like receptors (TLRs) 3, 7, and 8 in the endosomes, as well as cytoplasmic RNA sensors like retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5), the antiviral protein kinase R (PKR), and laboratory of genetics and physiology 2 (LGP2).[19,40,41] In light of these challenges and limitations, mRNA delivery vehicles should fulfill the following criteria: protect the mRNA cargo from degradation by nucleases, overcome the lipid bilayer, mediate efficient release from the endosomes, and prevent stimulation of the immune system by the foreign RNA.[42] In doing all this, the ideal carrier should be non-toxic and enable repeated dosing for the treatment of metabolic and/or hereditary diseases.

Based on the application of mRNA therapeutics, cells are either transfected *ex vivo* and then introduced back into the patient or mRNA is

delivered *in vivo* via different routes of application. The *ex vivo* method transfects the patient's cells with the desired mRNA by using physical methods like electroporation. This has been used in cancer immunotherapy,[43] genome engineering,[44,45] and genetic reprogramming approaches.[46–48] The second strategy involves *in vivo* delivery of mRNA to the patient. The methods that have been tested include intramuscular and intravenous (i.v.) delivery. While the former is mostly used for vaccination,[49,50] i.v. delivery has been used in studies addressing protein replacement.[51–54]

In this review, we focus on non-viral delivery systems for *in vivo* delivery of mRNA to the liver. Such delivery systems can be broadly categorized into two groups, namely, lipids or lipid-like materials and polymers.

Lipid-Based Delivery Systems

Cationic lipids form complexes with the negatively charged mRNA, called lipoplexes, which are then endocytosed by cells.[55] These carriers are highly effective, and commercially available transfection reagents like Lipofectamine messengerMAX and TransIT are widely used *in vitro*.[39,55–63] Unfortunately, the majority of the commercially available reagents either don't work *in vivo* or result in significant toxicity like liver damage and immunogenicity.[64,65] One of the underlying reasons behind this loss of activity *in vivo* could be positively charged character of the resulting mRNA-carrier complexes. Upon systemic delivery, such positively charged lipo- and poly-plexes get coated with the negatively charged serum proteins, thereby resulting in their rapid clearance from the circulation via the mononuclear phagocyte system.[66]

Lipid nanoparticles (LNPs) are lipid-based carrier systems that consist of a cationic lipid, a helper lipid, cholesterol, and polyethylene glycol (PEG).[12] These excipients form stable particles, which carry the mRNA in their inner core to protect it from degradation. LNPs are the most advanced delivery systems, and they were initially proven to be safe and efficient for siRNA delivery.[66–68] LNPs were investigated for mRNA delivery in a comprehensive study by Pardi et al.[69] In their study, the authors demonstrated the effect of administration routes on the distribution of mRNA-LNP complexes based on the lipid 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA). Whereas local expression was seen after subcutaneous, intramuscular, and intradermal injections of 0.005–0.250 mg/kg LNP-formulated mRNA, i.v., intraperitoneal, and to a lesser extent also intramuscular and intratracheal deliveries resulted in systemic mRNA trafficking and translation in the liver for up to 4 days.[69]

Another approach generated a diverse library of amino-polyesters formulated into LNPs, which were tested for mRNA delivery.[70] Kowalski et al.[70] i.v. applied 0.6 mg/kg mRNA, and they found differential distributions through lung, liver, and spleen, depending on the polymer composition. A recent publication by Jiang et al.[71] demonstrated physiological efficacy post-mRNA-LNP delivery in acute intermittent porphyria mice. Hepatic porphobilinogen deaminase (PBGD) production was observed as early as 2 h after i.v. injection of a dose of 0.5 mg/kg mRNA. Encouraging results in mice were further validated in non-human primates, where single or multiple i.v. doses of 0.5 mg/kg mRNA induced an increase in hepatic PBGD activity of roughly 80% at 24 h post-infusion.[71] Moreover, protein replacement of angiotensin-converting enzyme 2 in the liver and lungs of mice using 1 mg/kg mRNA-LNPs was shown by Schrom et al.[72]

Toxicity concerns might be alleviated by a study from Sedic et al.,[60] who evaluated the safety of modified mRNA encoding for human erythropoietin packaged in LNPs. Up to 0.3 mg/kg mRNA-LNP was applied two times weekly to rats and monkeys in repeated i.v. injections. Besides demonstrating the pharmacological activity of their approach, the authors also found the induction of interferon-γ production and liver injury in rats and splenic necrosis and lymphocyte depletion in monkeys upon mRNA treatment. However, these mild-to-moderate proinflammatory responses might be reducible by lowering the dose or decreasing the dose frequency. Together with the approval of Patisiran, which is an LNP-delivering siRNA, these data give evidence for the controllability of immunogenicity and safety concerns.[73]

Polymer-Based Delivery

Polymer-based materials are the second group of delivery systems. Examples include poly(amido-amine), poly-beta amino-esters (PBAEs), and polyethylenimine (PEI), with the latter being the most widely used. So far, PEI-conjugate-based delivery vehicles have mostly been used for mRNA vaccination approaches.[74,75] Poly (amido-amine)-based polyplexes have been studied for protein replacement approaches, e.g., to deliver erythropoietin-encoding mRNA.[76] Crowley et al.[77] showed that mRNA was successfully packaged into PEGylated polyacridine peptide polyplexes. These polyplexes have the proven ability to protect plasmid DNA in the circulation, but they can also stabilize mRNA by intercalative binding to double-stranded regions. *In vivo*, the polyacridine PEG-peptide mRNA reached a 15-fold increase of luciferase expression in the liver upon hydrodynamic dosing relative to polylysine PEG-peptide polyplexes. This study showed the applicability of PEGylated polyacridine peptide *in vitro* and *in vivo*, although the authors discussed that hydrodynamic dosing may not be usable for mRNA delivery to humans.[77]

Additionally, self-assembling block polymers consisting of an outer PEG shell and polymeric nanoparticles have been used for mRNA delivery in vaccination studies.[12] Moreover, latest technologies combine lipid and polymer particles to deliver mRNA. This was demonstrated in a recent study using a combination lipid-based and polymer-based delivery system, the hybrid mRNA technology delivery system comprising an LNP to protect the mRNA and a polymer micelle that targets hepatocytes and triggers an endosomal release of mRNA. Their mRNA encoding for ornithine transcarbamoylase (OTC) was applied i.v. into mice and reached an effective protein production specifically in the liver.[78] The efficiency of some of these mRNA delivery technologies that have been developed over

Table 1. An Overview of Preclinical Studies on Transcript Therapy for Certain Liver Diseases and Their Method of Delivery

Disease	Target	Delivery Method	Reference
Urea cycle disorder	*ornithine transcarbamoylase*	LNP	78,80
Crigler-Najjar syndrome type 1	*bilirubin-UGT*	LNP	51
Alpha-1 antitrypsin deficiency	*SERPINA1*	LNP	96
Thrombotic thrombocytopenic purpura	*ADAMTS13*	LNP	53
Glycogen storage disease type 1A	*glucose-6-phosphatase*	LNP	106
Acute intermittent porphyria	*PBGD*	LNP	71
Factor IX deficiency hemophilia B	*FIX*	C12-200 LNP LUNAR LNP	1,107

the past decade was tested in preclinical studies in different disease models. These studies are listed in Table 1 and discussed briefly below.

Urea Cycle Disorder

Single gene mutations in enzymes involved in the urea formation result in a buildup of intermediates of the urea cycle. Consequently, free ammonia, which is toxic to the tissues, is absorbed by the bloodstream. The failure of prescription treatments leaves liver transplantation as the only curative option. There is indeed a significant treatment opportunity for mRNA-based therapeutics to target the urea cycle disorders by delivering the appropriate mRNA. Prieve et al.[78] showed a preclinical proof of concept for OTC deficiency in a mouse model, where mRNA was efficiently delivered to the liver cells. These results are highly promising, as no toxicity-associated side effects were observed, even upon multiple-dose application.[79] Besides OTC deficiency, other urea cycle deficiencies, e.g., argininosuccinate synthetase deficiency, are also likely to be treatable using mRNA-based approaches. As described by Heartlein, Derosa, and Smith, argininosuccinate synthetase protein levels could be detected already after a single i.v. dose of the respective mRNA.[80] Its expression lowered the blood ammonia levels in mice back to the normal values.

Crigler-Najjar Syndrome Type I

Crigler-Najjar syndrome type 1 (CN1) is an autosomal recessive disease, affecting less than 1 in 1 million newborns worldwide.[81] Individuals carrying the mutation in the UGT1A1 gene, which produces dysfunctional bilirubin uridine diphosphate glucuronosyltransferase (bilirubin-UGT), develop hyperbilirubinemia and, consequentially, experience jaundice and irreversible brain and muscular damage. Symptom relief treatment involves blue light phototherapy[82] with a goal of breaking down bilirubin to a more soluble form, but the only successful long-term treatment is hepatocyte transplantation.[83–85] However, new treatment options are constantly being investigated, and adeno-associated virus (AAV) gene therapy is already undergoing phase I/II clinical trials.[86]

Since the disease is caused by a genetic mutation leading to the lack of an enzyme, it is an attractive target for liver-directed mRNA therapeutics to provide the correct copy of the impaired enzyme. An i.v. mRNA therapeutic delivering bilirubin-UGT encapsulated in LNP was successfully delivered *in vivo*, specifically to hepatocytes of Gunn rats (a rodent model of CN1 disease), with minimal uptake by other tissues and organs.[51] A modified mRNA was able to restore hepatic expression of UGT1A1 and, consequently, normalized the bilirubin pathway. The treatment, even after multiple doses, did not show toxic effects. Based on *in vivo* pharmacology data obtained from preclinical studies, LNP stability, mRNA cellular kinetics, and protein turnover, a quantitative system pharmacology model was developed. As a predictor of mRNA pharmacology, it opens a door for the design of the first-in-human clinical trials of UGT1A1-mRNA treatments for CN1.[51]

Alpha-1 Antitrypsin Deficiency

Caused by the mutation in *SERPINA1* gene alleles, alpha-1 antitrypsin deficiency (AATD) is a rare hereditary disease affecting the liver and lungs of patients. Alpha-1 antitrypsin (AAT) is a serine protease inhibitor that normally protects tissues from enzymatic proteolytic damage and is released upon neutrophilic inflammation. To slow down pulmonary destruction, patients are offered i.v. administered alpha-1 antitrypsin,[52] derived from the donor's blood plasma. Despite its biochemical efficiency, many European and USA countries have not accepted it as a treatment option due to a lack of clinical effectiveness. Therefore, many alternative options are being investigated, such as autophagy-enhancing drugs;[87] induced pluripotent stem cell (iPSC)-genome editing based on zinc-finger nucleases,[88] transcription activator-like effector nucleases (TALENs),[89] and the CRISPR/Cas9 system;[90,91] gene replacement therapy based on viral[92] and non-viral[4,93] mediators; microRNA (miRNA) for gene silencing; and mRNA as a transcript therapy.[94–96] In a recent study by Connolly et al.,[96] transfection of patient fibroblasts and hepatocytes with AAT-encoding mRNA resulted in significant secretion of AAT protein in the cell culture supernatant. An i.v. injection of mRNA LNPs in mice resulted in the efficient delivery of the therapeutic mRNA to liver and lungs. Both the tissues were positive for AAT protein and mRNA, thereby confirming efficient delivery and translation of the mRNA molecule.

Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is a rare, fatal autosomal recessive disease affecting 3–11 individuals per million people per year.[97] In 5% of cases, the disease is caused by hereditary mutations in a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13)-encoding gene.[98] In the case of ADAMTS13 enzyme deficiency, thrombi form and circulate around the body, systematically damaging the heart, kidneys, brain, and nervous system. Although the disease affects primarily the cardiovascular system, ADAMTS13 is produced in hepatocytes and secreted into the circulation. Therefore, treatment options aiming at enhancing and/or supplementing ADAMTST13 protein production are targeted to the liver. Many novel gene therapeutic options have

been investigated in preclinical studies.[99–101] In the recent work by Liu-Chen et al.,[53] a full-length optimized ADAMTS13 mRNA was synthesized and encapsulated into LNPs. Upon a single i.v. administration into ADAMTS13-deficient mice, 20% of the normal enzymatic activity was detectable in mouse circulation and maintained for 48 h, showing that hepatocytes successfully expressed and secreted ADAMTS13. This therapeutic approach could provide a treatment for patients with a steady supply of secreted enzyme for a few days while still waiting for liver transplantation.[53]

Glycogen Storage Disease Type 1A

Glycogen storage disease type 1A (GSD1) is a metabolic autosomal recessive disorder, caused by the malfunction of the catalytic subunit of glucose-6-phosphatase (G6P), an enzyme that yields free glucose by hydrolysis of G6P. Since the gluconeogenesis takes place mainly in the liver, it is the first organ to be affected by the disease. The symptoms include severe hypoglycemia, renal disease, hypertriglyceridemia, and anemia.[102] While the patients with end-stage renal failure are treated with kidney transplantation, there is no option to cure the metabolic abnormalities.[103] Recently, a liver-directed gene therapy using adenoviral vectors was shown to potentially correct G6P gene in deficient mice, resulting in 19% restoration of G6P function and normalization of glucose, lipid, and uric acid levels.[104]

A similar therapy based on adeno-associated virus vectors encoding G6P was applied to canine puppies, and it restored about 50% of normal G6P liver expression.[105] However, due to inherent risks from random insertions and a risk of cancerous effects, mRNA therapy would be a potential strategy to reverse the hepatic abnormalities associated with GSD1. Roseman et al.[106] demonstrated that a single administration of LNP-G6P mRNA successfully corrects the G6P deficiency in G6P$^{-/-}$ mice, which established normal blood glucose levels and reduced hepatic triglycerides and glycogen. Using mRNA encoding a protein-engineered variant of G6P, 2%–4% of G6P activity was detectable up to 12 days after a single application. In earlier studies, it was reported that a minimum of 2% of wild-type (WT) G6P activity is required to maintain glucose homeostasis and prevent hepatocellular adenoma.[54] This study further establishes mRNA as a potent therapeutic molecule for the treatment of inherited liver disorders.

Acute Intermittent Porphyria

Deficiency of PBGD, an enzyme involved in the heme biosynthesis, is caused by a genetic mutation of the *PBGD* locus. Patients experience neurovisceral attacks associated with a buildup of neurotoxic porphyrin precursors. For the moment, the effective treatment for recurrent symptoms is hemin supplementation; but, with its adverse long-term side effects like an iron overload in the liver, it is far from a sustainable therapy. As a possible treatment, developed by Jiang et al.,[71] mRNA encoding for PBGD was encapsulated in LNPs and addressed to the liver of animal models (mice and rabbits) of the disease. PBGD was observed in the liver just 2 h after the mRNA application, and it rapidly normalized mice's porphyrin precursors in the urine. The translatability toward clinical trials was demonstrated by an experiment in non-human primates. The tolerability of the treatment after single and repeated doses was confirmed in all three *in vivo* models. This therapeutic approach addresses the sustained correction of symptoms, and, therefore, it prevents the need for more invasive treatments, such as liver transplantation.

Factor IX Deficiency Hemophilia B

This blood-clotting disorder is caused by a deficiency of factor IX (FIX), a serine protease that then disrupts the coagulation cascade. The mutation in the *FIX* gene is recessively inherited by the X chromosome, therefore mostly affecting male family members through female carriers. The disease is manifested through long, internal bleeding and easy bruising. The prophylactic treatment is an i.v. application of purified FIX along with blood transfusion, but it must be given several times a week. The first successful encapsulation and delivery of mRNA constructs to the liver using C12-200-based LNP technology was presented by DeRosa et al.[107] Hepatocyte deposition and detection of active mRNA were observed for both of the tested transcripts, human EPO and hFIX mRNA, resulting in high and sustained protein production for up to 1 week post-injection. Therapeutically relevant amounts of human FIX (hFIX) protein were achieved upon a single i.v. dose of hFIX mRNA-loaded LNPs in mice. In addition, therapeutic value was established within a hemophilia B (FIX-knockout [KO]) mouse model by demonstrating a marked reduction in hematocrit loss following injury (incision) to FIX KO mice.

Therapeutic levels of FIX were also achieved in a preclinical mouse model of hemophilia B by an effective delivery of mRNA to the liver via LUNAR LNPs.[1] The clotting defect was alleviated already 4 h post-application, and circulation levels of hFIX were present for up to 6 days. A benefit of transcript therapy compared to any other treatment (e.g., recombinant protein) is that FIX undergoes significant post-translational modifications, such as O- and N-linked glycosylation, β-hydroxylation, and γ-carboxylation, which take place in the liver. As mRNA is directly delivered to hepatocytes, a natural niche of FIX production and secretion, the treatment offers a viable therapeutic alternative.

Outlook

The identification of liver diseases susceptible to mRNA protein replacement therapy has led to an expansion of research in the field of targeted hepatic mRNA delivery. However, some technical limitations still represent the challenges ahead. In this review, we have focused on mRNA delivery to the liver in general without addressing co-delivery to other tissues (non-targets). Keeping in mind the numerous hurdles on the way of mRNA from laboratory to the ribosomes in the target cells, ideal mRNA delivery vehicles should not only protect the mRNA cargo from degradation by nucleases and overcome the cellular plasma membrane but also ensure efficient release from the endosomes without stimulating the innate immune responses.

One of the most critical obstacles for clinical translation of mRNA therapeutics is the selective *in vivo* delivery to target cells and tissues,

thereby minimizing the off-target effects. Most studies to date have focused on disease modalities that do not require a repetitive application or high dosage, which in turn result in the limited exposure of the tissues to the synthetic, unnatural lipids. However, the needed dosing scheme is most likely to be dependent on protein half-life (the therapeutic molecule) and its minimum levels needed for physiological response rather than the mRNA itself. mRNA therapeutics encoding for proteins with a longer half-life and needed in minuscule amounts are likely to offer a broad therapeutic window with a less frequent application. Future studies using specific disease models investigating pharmacokinetic (PK)/ pharmacodynamic (PD) dynamics in detail will be informative about the frequency of application and therapeutic window of the mRNA molecule.

Another critical parameter is going to be the route of application. At present, the method of choice for hepatic mRNA delivery is an i.v. infusion, which requires the patient's compliance and active participation of the medical practitioners. Delivery technologies offering alternative routes of potent and selective liver delivery are most likely to expand the repertoire of liver diseases amenable to such an approach. For example, direct liver injection has been used in a clinical trial (ClinicalTrials.gov: NCT01437007) for delivering siRNA in liver cancer patients. Using magnetofection, Morishita et al.[108] reported enhanced delivery of plasmid DNA to mouse liver. In a recent study by Pardi et al.,[69] delivery of mRNA-LNPs via an intraperitoneal route also led to liver transfection. Further studies are needed to test the applicability of these methods to deliver mRNA therapeutics in large, clinically relevant animal models. Going by the development of the past decade, the recent preclinical studies in disease models, and the approval of LNP RNAi drug Patisiran, one can be optimistic about hepatic transcript therapies reaching the clinic and serving the ultimate goal of healing the patients.

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