Hospital-Based RNA Therapeutics

Tulsi Ram Damase, Roman Sukhovershin, Min Zhang, Daniel L. Kiss, and John P. Cooke

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Abstract Hospital-based programs democratize mRNA therapeutics by facilitating the processes to translate a novel RNA idea from the bench to the clinic. Because mRNA is essentially biological software, therapeutic RNA constructs can be rapidly developed. The generation of small batches of clinical-grade mRNA to support IND applications and first-in-man clinical trials, as well as personalized mRNA therapeutics delivered at the point-of-care, is feasible at a modest scale of cGMP manufacturing. Advances in mRNA manufacturing science and innovations in mRNA biology are increasing the scope of mRNA clinical applications.

Keywords Messenger RNA · Hospital-based mRNA therapeutics · Circular mRNA · Self-amplifying mRNA · RNA-based CAR T cell · RNA-based gene-editing tools

1 Introduction

A therapeutic revolution is underway. It is now quite clear that message RNA (mRNA) therapies will become a major therapeutic armamentarium against infectious diseases, as they provide a platform for a flexible and rapid response to

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pandemics. However, the scope of mRNA therapeutics is much broader. Because mRNA is essentially biological software, one can rapidly write the code for any protein and thereby create vaccines against infectious diseases, cancer, or any pathogenic protein where an immunogenic response is desired. One can also generate mRNA constructs encoding proteins that are deficient, such as in the congenital absence of a metabolic enzyme. In addition, it is possible to use mRNA to enhance cell therapies, or to biologically modify scaffolds used in regenerative medicine. The excitement about mRNA therapies is reflected in the high valuations of the companies that are early movers in this field, such as Moderna, BioNTech, and Curevac, each of which also has partnerships with large pharmaceutical companies. However, the future of mRNA therapies will not be the exclusive province of big pharma. Because mRNA therapies can be rapidly generated with manufacturing processes that are less complicated than recombinant proteins and have a simpler regulatory roadmap than new chemical entities, it becomes possible for small companies and academic groups to participate in therapeutic development and clinical application. Furthermore, hospital-based programs can fill gaps in personalized therapies and rare diseases that are not addressed by large pharmaceutical programs.

2 A Role for Hospital-Based Drug Development

Development of therapeutics within academic hospitals can expedite the development of novel and personalized therapies, particularly for rare diseases, and speed their entry into clinical trials. Such programs combine unique scientific expertise in certain field or technology with the translational and clinical infrastructure available on site. Notably, hospital-based programs can bring therapeutics from bench to bedside (Damase et al. 2021) and can facilitate the development of personalized treatments for rare diseases. With respect to nucleic acid therapeutics, Nationwide Children's Hospital, St. Jude Children's Research Hospital, and Boston Children's Hospital are examples of successful hospital-based gene therapy programs.

Nationwide Children's Hospital is known for Zolgensma and Golodirsen—gene therapies that have transformed care of patients with spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), respectively. SMA patients have a defective survival motor neuron 1 (SMN1) gene. A deficiency of survival motor neuron (SMN) protein leads to the death of motor neuron cells that control muscle function, thus affecting the patient's ability to move, swallow, and breathe. Zolgensma delivers a fully functional copy of the human SMN1 gene into the target motor neuron cells through a viral vector AAV9. A single infusion of Zolgensma generates sufficient SMN protein in motor neurons to improve muscle movement and function (Caccomo 2019; Waldrop et al. 2020; Damase et al. 2021).

Patients with DMD have a mutation in the gene that encodes dystrophin. The latter protects muscle cell membranes, and its loss causes myofibril damage and muscle atrophy in early childhood, dramatically shortening life expectancy. Golodirsen is an antisense oligonucleotide that binds to DMD pre-mRNA and affects its splicing

to skip the defective exon and generate a functional dystrophin protein (Kahn 2019; Damase et al. 2021; Scaglioni et al. 2021).

In St. Jude Children's Research Hospital, a gene therapy to treat severe combined immunodeficiency (SCID-X1) was developed. SCID-X1 is caused by mutations in the gene encoding IL2RG protein on the X chromosome, which is essential for immune system functioning. Patients without this protein have very few natural killer and T cells, as well as nonfunctional B cells. To correct IL2RG deficiency, the bone marrow stem cells are obtained from the patient and IL2RG gene is delivered ex vivo with lentiviral vectors. Genetically modified cells are infused back into the patient after bone marrow ablation (Mamcarz et al. 2019; Damase et al. 2021). Eight patients were treated so far and all tolerated and responded well to this therapy.

Boston Children's Hospital has pioneered the development of personalized gene therapy for a 7 year-old girl diagnosed with a novel mutation in CLN7/MFSD8, a subtype of Batten's disease. The mutations in CLN7 gene disrupt MFSD8 protein synthesis. Although its exact function is unknown, MFSD8 is believed to be involved in transport across the lysosomal membrane. The patient presented with an insidious onset of impaired vision, ataxia, seizures, and developmental regression. The presence of characteristic lysosomal inclusions on skin biopsy suggested the diagnosis which was confirmed by genetic testing. Standard genetic testing revealed heterozygosity for a single pathogenic mutation in CLN7. Because the disease is autosomal recessive, the suspicion that there was a second mutation was confirmed by whole-genome sequencing. Specifically, an SVA retrotransposon insertion was found that resulted in a cryptic splice acceptor site (i6.SA) that caused a mis-splicing of exons 6 and 7. To prevent mis-splicing, the antisense oligonucleotide milasen targeting the i6.SA site was designed and tested initially on the patient's fibroblasts in vitro. Milasen has the same backbone and sugar chemistry modifications as nusinersen, an FDA-approved drug for SMA, which changes the splicing pattern of SMN2 to resemble SMN1. In culture, milasen boosted the normal:mutant splicing ratios by a factor of 2.5–3. Its safety was confirmed in toxicity studies, and clinical trial was initiated within 1 year after the first contact with the patient, during which a clinical improvement was noted (Kim et al. 2019; Damase et al. 2021).

At Houston Methodist Hospital in the Texas Medical Center, we have established a hospital-based program for mRNA therapeutics. mRNA-based therapeutic technology is a disruptive technology as it facilitates rapid development and clinical translation of novel solutions for previously "undruggable" diseases. It can be more rapidly deployed by academic groups and small biotech start-ups. However, most of these small entities lack the crucial infrastructure required to bring their transformative therapy from the lab bench to the clinic. They need partners with expertise in RNA manufacturing, formulation, preclinical and clinical testing. Our program at Houston Methodist Hospital provides a translational pipeline for small entities such as biotechnology start-ups or academic groups with great ideas about RNA therapeutics. Houston Methodist Hospital (HMH) made a strategic investment in its research institute to build the infrastructure necessary to support preclinical and clinical translation of novel therapeutics. The RNAcore facility, a central pillar of our program, is housed within the research institute and is managed by a team of

scientists with expertise in engineering and manufacturing mRNA constructs for the scientific community. Within past 5 years, we have generated more than 100 unique constructs for more than 40 collaborators. Funded in part by the NIH and the Cancer Prevention and Research Institute of Texas (CPRIT), the RNA Core facilitates the development of RNA therapeutics globally, with a particular interest in supporting cancer research in Texas.

Our program encompasses all essential competencies that are required for development and translation of mRNA-based drugs (Fig. 1). Our RNA biology and bioinformatics groups innovate RNA design to enhance the stability and translational efficiency of our RNA constructs. Our nanomedicine group helps in the generation

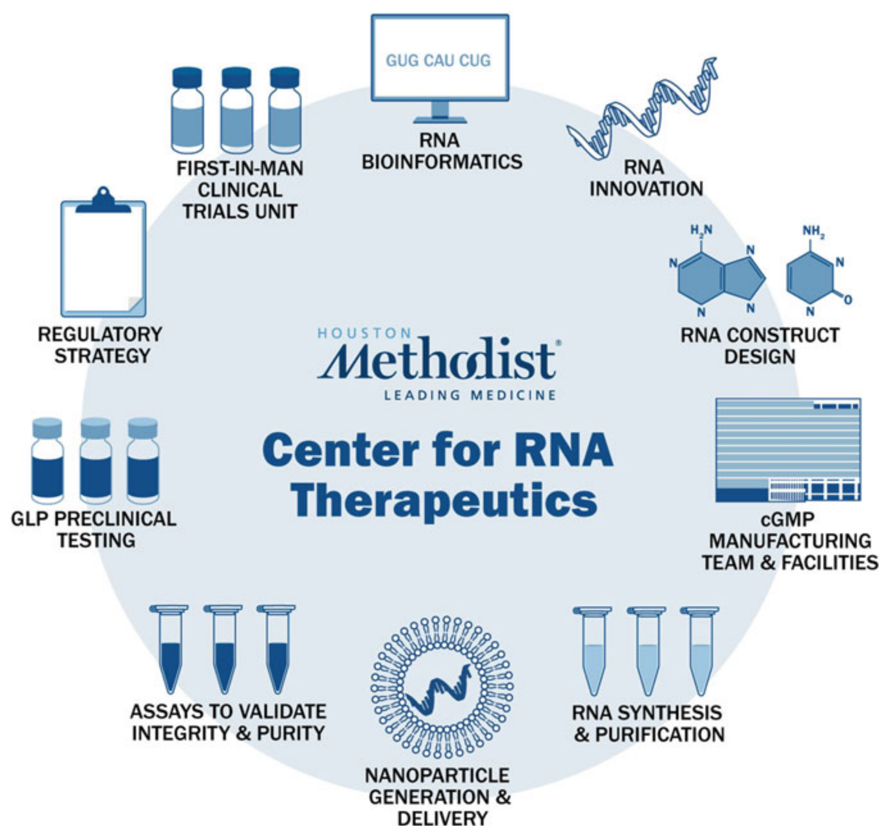

Fig. 1 Schematic of Houston Methodist Hospital center for RNA therapeutics. As typical of a hospital-based therapeutic program, we have a deep fund of knowledge in our therapeutic arena, and facilities for synthesizing clinical grade materials. We have expertise in RNA bioinformatics and innovation; with proprietary manufacturing methods for the synthesis, purification, validation, and encapsulation of mRNA; facilities and personnel for GLP preclinical studies in preparation for an IND; clean rooms and cGMP processes and personnel for generating clinical-grade mRNA and LNPS; a phase 1 unit for first-in-man clinical trials; and a large hospital system for doing later stage clinical trials

of lipid formulations and fabrication of lipid nanoparticles to deliver RNA. The Office of Translational Production and Quality (OTPQ) guarantees quality products for patients and provides in-house cGMP facilities, with clean rooms and calibrated equipment for manufacturing cGMP-grade RNA and lipid nanoparticles. The in-house quality resources lead to fast and cost-effective GLP-grade test and release of mRNA-based products for clinical use. An Office of Regulatory Affairs aids with the development of the regulatory roadmap and interactions with regulatory bodies. Our in-house Comparative Medicine Program assists with preclinical animal studies, including GLP studies for IND applications. Our Clinical Trial Center has skilled nurses and physicians that perform the first-in-man clinical trials.

Our hospital-based program can produce small batches of cGMP RNA therapeutics to support early phase clinical trials. For later stage clinical trials and commercialization, the project is carried forward by our partner VGXI Inc., based just outside of Houston. Together with VGXI we have scaled our manufacturing processes in their facility so that there is a seamless transition for mRNA drugs from small batch manufacturing for GLP preclinical studies and early phase clinical trials, to the large batch manufacturing that is required for products that are entering later stage clinical trials (Fig. 2). VGXI has prior expertise in large batch manufacturing of DNA therapies which facilitated the partnership. Currently, there is no other academic program

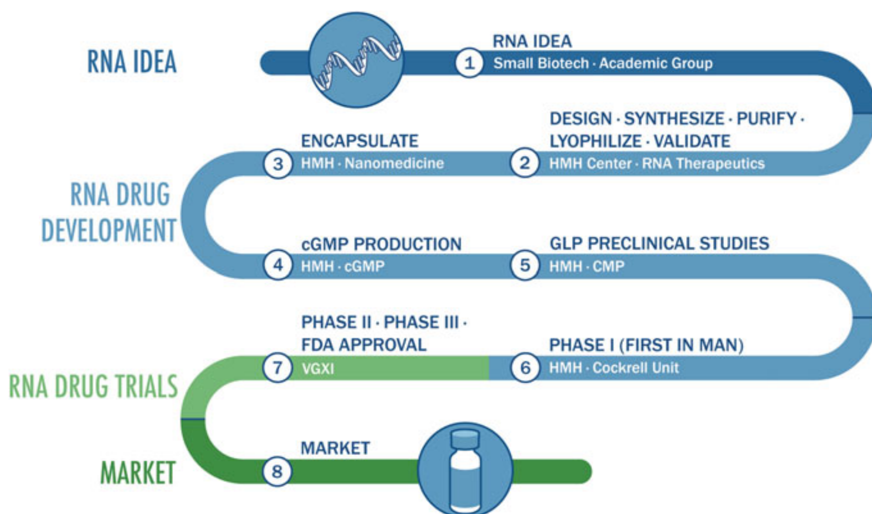

Fig. 2 Roadmap to the clinic. Our intent is to assist academic groups and small companies with a great RNA idea translate their technology to the clinic. Many small groups do not have some of the core competencies to traverse the gap from bench to bedside. We have processes and personnel to synthesize, purify, encapsulate, and validate the integrity, purity and strength of the product, as well as the facilities and experience for preclinical studies and early clinical trials. A corporate partner (VGXI Inc, Woodlands TX) uses our scaled-up processes for large batch manufacturing to support later stage clinical trials and commercialization

in the USA with comparable capabilities. However, we anticipate that the therapeutic revolution in mRNA, together with advances in manufacturing technology, and the need for personalized RNA drugs, will create the demand for such centers. These regional centers will provide a pathway for academic investigators and start-ups to translate their novel RNA therapeutics ideas from the bench to the bedside (Damase et al. 2021). Thus, the generation of small batches of clinical-grade mRNA to support IND applications and first-in-man clinical trials, as well as personalized mRNA therapeutics delivered at the point-of-care, is feasible at a modest scale of cGMP manufacturing within a hospital-based program.

3 Manufacturing of mRNA

In theory, it is possible to generate an RNA construct for any protein of interest. The production of mRNA is relatively straightforward, fast, and robust process. Moreover, a generic production workflow can be used to make broad variety of RNA constructs, as simple changes in RNA sequence will not affect the methods used in manufacturing.

mRNA for therapeutic applications is usually transcribed in vitro (IVT) from the DNA template using a bacteriophage DNA-dependent RNA polymerase (T7, T3, or SP6) and ribonucleotide triphosphates (NTPs) (Pardi et al. 2013). In a DNA template, a protein-encoding sequence should be placed downstream from the promoter sequence and flanked by 5′ and 3′ untranslated regions (UTRs). Kozak sequence significantly affects expression and should be added in front of protein-coding sequence (Kozak 1987). The poly A tail-related sequences can be included after 3′UTR (Pardi et al. 2013) to either transcribe the tail from the template directly or perform enzymatic polyadenylation after transcription (Pardi et al. 2013). If a plasmid is used as a template, then a unique restriction site should be incorporated downstream of the poly A tail-related sequence followed by T7 terminator sequence. This restriction site aids in cutting (or linearizing) the template at a specific site to ensure the polymerase terminates transcription to generate mRNA of defined length (Fig. 3). DNA templates can also be prepared by polymerase chain reaction (PCR).

To ensure RNA translation, 7-methylguanylate cap must be incorporated into mRNA either post-transcriptionally with vaccinia capping enzyme (Martin and Moss 1975) or co-transcriptionally through the addition of an anti-reverse cap analog (ARCA) or CleanCap reagent (Henderson et al. 2021). Different modified NTPs, such as pseudouridine or 5-methyluridine, may be incorporated into mRNA construct by adding them to IVT reaction mixture. After RNA is transcribed, enzyme DNase can be used to remove template DNA from the reaction mixture (Green and Sambrook 2019).

Replacing some standard RNA bases with the modified nucleosides mentioned above eliminated much of the innate immune stimulatory response that was triggered by IVT RNA. However, a low-level induction of interferons (IFNs) and inflammatory cytokines still remained (Karikó et al. 2011). These immune responses

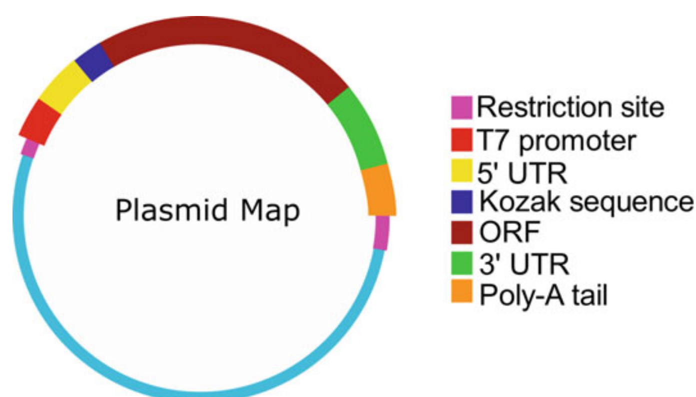

Fig. 3 Typical plasmid map. The typical DNA plasmid used in manufacturing will incorporate many restriction sites so as to easily incorporate different open reading frames encoding the protein of interest. The DNA plasmid is amplified using bacterial fermentation, purified, and linearized for the in vitro RNA transcription. 5′UTR = 5′ untranslated region. 3′UTR = 3′ untranslated region. ORF = open reading frame

were triggered by trace contaminants including double-stranded RNA (dsRNA), uncapped RNA, and short abortive RNAs which occur as byproducts of high-yield IVT reactions (Karikó et al. 2011). When combined with nucleoside modifications mentioned above, the efficient removal of these trace contaminants yields low-to-non-immunogenic IVT mmRNAs.

Purification methods have reduced the immunogenicity of IVT mmRNAs and have enabled the development of mRNA therapeutics that will be applicable to many diseases. Precipitation (Henderson et al. 2021) and liquid chromatography-based methods (Karikó et al. 2011) can be employed to purify RNA from enzymes, free NTPs, residual DNA, truncated RNA fragments, and double-stranded RNA. At small scales, preparative polyacrylamide gel electrophoresis is a powerful and commonly used tool to separate the desired RNA products from abortive products or other impurities (Green and Sambrook 2021). However, this method is only suitable for short RNA oligonucleotides or small coding or non-coding RNAs (up to a limit of ~ 500 nucleotides) (Summer et al. 2009). Further, as gel purification methods are both inefficient and are not scalable to large production lots, other purification alternatives were needed for mmRNA therapies. Fast-performance liquid chromatography (FPLC) is reported to efficiently remove small abortive RNAs but is limited in its ability to distinguish RNA molecules with similar size (McKenna et al. 2007). High-performance liquid chromatography (HPLC) has also been identified as an easily performed and scalable method that removes multiple contaminants from IVT mRNAs (Karikó et al. 2011). For larger scale manufacturing, methods that avoid the use of flammable organic solvents are preferred. These methods may include sorbent-based or nanochanneled monolith affinity chromatography, ion exchange and size exclusion, reversed phase chromatography, or reversed phase ion-paired chromatography. Purified RNA products must be stored frozen (preferably at − 80 °C),

unless lyophilized (Crommelin et al. 2021). It is critical to maintain an RNase-free environment while manufacturing RNA.

Quality control (QC) is an essential component of RNA manufacturing processes. The following quality attributes are commonly assessed in finished RNA products: identity (sequencing), concentration (UV absorbance), integrity (electrophoresis; RT-PCR), capping efficiency (LC–MS and others), residual protein (colorimetric methods), residual DNA (qPCR), double-stranded RNA (blotting, ELISA), residual solvents/buffer components (if used for purification; methods vary).

4 Innovations in mRNA Therapeutics

A greater understanding of RNA biology contributed to the emergence of RNA therapeutics. One of the major obstacles to the field was that synthetic RNA molecules triggered robust immune responses when introduced into mammalian systems (Alexopoulou et al. 2001; Damase et al. 2021). These immune responses thwarted the development of mRNA-based therapies, especially protein replacement strategies for many years (Karikó et al. 2005; Damase et al. 2021). A seminal insight that advanced the field was the finding that in vitro transcribed (IVT) mRNA activated innate immune signaling through toll-like receptor signaling (TLR3/7/8), retinoic acid-inducible gene I protein (RIG-1), or melanoma differentiation-associated protein 5 (MDA5) (Alexopoulou et al. 2001; Heil et al. 2004; Yoneyama et al. 2004, 2005). Subsequently, the development of more efficient capping methods, the incorporation of modified nucleosides, as well as specialized purification protocols, were found to substantively reduce the high immunogenicity of IVT mRNAs (Karikó et al. 2005; Damase et al. 2021).

4.1 Reducing Immunogenicity

Different modified RNA bases have been assessed for their potential to reduce the immunostimulatory side effects inherent within IVT mRNA. Previous studies reported that modified nucleosides including pseudouridine (Ψ), 5-methylcytidine (m5C), 5-methyluridine (m5U), N6-methyladenosine (m6A), 2-thiouridine (s2U), or N1-methylpseudouridine (m1Ψ) eliminate the majority of TLR stimulation (Karikó et al. 2005). Hartmann et al. also demonstrated that mRNAs incorporating both Ψ and s2U also suppressed or evaded RIG-1 signaling (Hornung et al. 2006).

Of these modifications, Ψ and m5C are the most commonly used to generate modified messenger RNA (mmRNA). The breakthrough experiments were performed by Kariko and Weissman and showed that incorporation of Ψ not only reduces recognition by innate immune sensors but it also increases the translational capacity of the mmRNA (Karikó et al. 2008, 2011). Recently, the m1Ψ substitution has been shown to outperform other modified nucleosides via a more efficient immune response

escape combined with a further increase in translational output (Parr et al. 2020). Consequently, Pfizer and Moderna replaced 100% of the uridine residues in their COVID-19 mRNA vaccines (Pfizer/BioNTech (BNT162b2) and Moderna's mRNA-1273) with m1Ψ to improve the performance of their vaccines (Jackson et al. 2020; Polack et al. 2020; Corbett et al. 2021). A combination of s2U and m5C was also found to reduce the immune signaling through abrogating mRNA interaction with TLRs and RIG-1 (Hornung et al. 2006). mmRNA therapeutic candidates containing this combination of modified bases have been employed to restore the expression of a deficient gene in a mouse model (Kormann et al. 2011).

4.2 Cell-Specific/Tissue-Specific Delivery and Translation

A current challenge in RNA therapeutics is to attain cell-specific or tissue-specific delivery and translation. Almost certainly, just as a combination of approaches was needed to effectively reduce the inflammatory properties of IVT mRNAs, a combination of approaches may address the need for tissue-specific expression.

Currently, lipid nanoparticles (LNPs) are most widely used as a delivery vehicle for mRNA. Indeed, the two mRNA vaccines for SARS-CoV-2 that are currently approved for use are each encapsulated in LNPs. The LNPs stabilize the mRNA by reducing its interaction with water, or with RNAases that are ubiquitous in the environment. As a vaccine, the mRNA LNP is simply injected into the deltoid muscle (as with the SARS-CoV-2 vaccine) or subcutaneous tissue, where it can be taken up by resident antigen-presenting cells to initiate an immune response. However, for delivery to other organs, the situation is complicated by the fact that LNPs circulating in the blood are avidly taken up by the reticuloendothelial system, i.e., the liver and the spleen. For this reason, hepatic diseases are an excellent target for current mRNA therapies. Other organs are more difficult to target. Approaching is to modify the LNPs with tissue-specific "addresses" such as antibodies directed against a tissue-specific antigen (Cheng et al. 2020; Molinaro et al. 2020). In a related approach, biomimetic particles that incorporate membrane proteins from leukocytes have been shown to target inflamed tissues (Cheng et al. 2020; Molinaro et al. 2020). These so-called leukosomes accumulate at sites of inflammation, where the endothelium is activated to express ligands that bind leukocyte counter-ligands that have been incorporated into the LNPs. Leukosomes have been shown to deliver the immunosuppressant rapamycin to the atheromatous aorta of hypercholesterolemic mice, to reduce the proliferation of macrophages in the vessel wall (Christian et al. 2020).

Another strategy to achieve cell-specific translation is by designing an mRNA construct so that it is preferentially expressed in a tissue. For example, one can include tissue-specific microRNA (miRNA) recognition elements in the mRNA drug. It has been showed that, by incorporating into the 3′ UTR a cardiomyocyte-specific recognition element, miRNA-induced degradation of their RNA construct in cardiomyocytes provided for preferential expression in non-cardiomyocytes (Magadum et al. 2020).

Hewitt et al. also included a miR-122 binding site into the 3′UTR of their therapeutic IL-12 mRNA to prevent the IL-12 protein production in hepatocytes without impairing expression in cancer cells (Hewitt et al. 2020). Importantly, the inclusion of miRNA binding sites is a negative expression strategy. Simply, this means that the IVT mRNAs are actively degraded in off-target tissues (those with high levels of the miRNA whose sites have been added to the 3′UTR of the IVT RNA). While early studies show that this strategy can be effective in achieving tissue-specific expression, it is limited by the miRNA profiles of targeted tissues.

Trigger RNAs (trRNA) represent another early stage innovation that could be developed further to introduce more selectivity and aid with the targeting of mRNA therapies (Green et al. 2014; Zhao et al. 2021). Simply, trRNAs are trans-acting RNA sequences that recognize and de-repress the translation blockade caused by adding a toehold regulatory RNA sequence prior to the coding sequence of an mRNA (Green et al. 2014; Zhao et al. 2021). Currently designed trRNA sequences work in concert with IRES sequences, so it remains to be seen if trRNAs can effectively regulate open reading frames driven by cap-dependent translation (Zhao et al. 2021). Furthermore, current designs call for this system to work in trans where the presence of two exogenous RNAs in the same cell would allow the expression of the protein of interest (Green et al. 2014; Hanewich-Hollatz et al. 2019). As independent delivery of the trRNA and its target are required for the regulation to be fully effective, two different RNAs would be required to target the same cells to confer the desired regulation of translation.

4.3 Increasing mRNA Stability and Expression

The comparatively short lifespan of mRNA and mmRNA in cells remains a limiting factor for the utility of mRNA therapeutics. Limited mRNA lifespans may require high doses of mRNA (which increases the risk of adverse effects, as well as the cost of goods) or a repeated dosing schedule (increasing the expense of the therapy) required for clinical effectiveness. Such regimens are often incompatible with long-term treatment strategies required for many clinical indications.

One approach to increase mRNA longevity derives from the mechanisms by which Zika virus exoribonuclease-resistant RNAs (xrRNAs) function in infected cells (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). Simply, xrRNA sequences fold into a stable pseudoknot structure that confers considerable exoribonuclease-resistance to the RNAs bearing them (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). One may surmise how the addition of xrRNA sequences to the 5’UTR could improve the lifespan of a therapeutic mRNA in vivo. However, the ability of cells to robustly translate mRNAs containing xrRNA sequences in their 5′ UTRs remains unknown, although their presence exclusively in viral 3′ UTRs hints that they could inhibit traditional cap-dependent translation mechanisms (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). Further, since xrRNA sequences confer their functions by forming either tight

secondary structures and modified RNA bases often disrupt RNA base-pairing, the compatibility of xrRNA sequences with mmRNA therapeutics remains to be seen (Wesselhoeft et al. 2018; Mauger et al. 2019).

A very promising approach is to circularize the mRNA (circRNA) so that it is resistant to degradation. The promise of using circRNAs with an incorporated internal ribosome entry site (IRES) as a durable protein expression system has been known for decades (Collett and Faras 1976; Puttaraju and Been 1992; Ford and Ares 1994; Beaudry and Perreault 1995). The increased stability of circRNAs, also known as endless RNA, is mostly conferred by their circular structure. Simply, most cellular RNases attack and degrade RNAs by recognizing an unprotected 5′ or 3′ end (Yang 2011). Since circRNAs are continuous, they lack traditional RNA ends and evade most mechanisms that would degrade them resulting in an increased lifespan (compared to linear mRNAs) in vivo. Furthermore, with this increased RNA lifespan, a protein encoded by an IRES-initiated coding region within a circRNA will have a sustained expression pattern compared to a traditional mRNA. The biggest recent advance was made by the Anderson lab when they devised an efficient method to generate circRNAs of sufficient size to hold the open reading frame of a desired protein (Wesselhoeft et al. 2018, 2019). Their convincing findings show prolonged expression of different proteins from circRNAs in vivo (Wesselhoeft et al. 2018, 2019). Importantly, their method also succeeded in generating large circRNAs, including one encoding Cas9, which was over 4.9 kilobases (Wesselhoeft et al. 2018). Further, their subsequent work showed that their circRNAs were translated in vivo (Wesselhoeft et al. 2019). Indeed, several companies have capitalized on these properties and are developing different circRNA therapies.

Another approach lies in self- and trans-amplifying RNAs. In vivo replicating RNA technologies have been under development for many years (Geall et al. 2012; Bogers et al. 2015). These technologies consist of two components that can be contained either on a single or two RNAs. The first component is an open reading frame for the RNA-dependent RNA polymerase (RdRp) machinery of a virus, usually either Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEEV), or a chimera of VEEV and Sindbis virus replicons (VEE-SINV) (Blakney et al. 2021). Once those proteins are circulating in a cell, they can recognize the second component, which incorporates an RNA sequence encoding the open reading frame of the desired protein (Geall et al. 2012; Bogers et al. 2015).

Currently, two main types of replicating RNAs are being tested (Blakney et al. 2021; Bloom et al. 2021). First, self-amplifying RNAs (saRNA) are most similar to their parental viral sequences and have been under development for about a decade (Geall et al. 2012). As their name implies, saRNAs are single RNA transcripts that encode the viral RdRp machinery, RNA regulatory sequences that recruit the RdRp, and the open reading frame for the desired protein in a single RNA (Blakney et al. 2021; Bloom et al. 2021). Since saRNAs can contain five open reading frames, they are much longer than a traditional therapeutic mRNAs. As they are often exceed 10 kilobases, the large size of saRNAs does pose further challenges with manufacturing and packaging, but they have proven effective in limited studies (Geall et al. 2012; Bogers et al. 2015).

Trans-amplifying RNAs (taRNAs) are the second class of replicating RNA therapies in development (Blakney et al. 2021; Bloom et al. 2021). taRNAs work almost identically to saRNAs, but they are comprised of two distinct RNA strands that can be co-packaged into one delivery vehicle. Conceptually, and in practice, this change makes taRNAs a much more versatile platform technology with certain inherent advantages. First, with current technologies, manufacturing two ~ 5–6 kilobases RNAs is much simpler than efficiently manufacturing a single 10–12 kilobases RNA. Second, the modular design of taRNAs also offers manufacturers the ability to manufacture large lots of a single replicase RNA while allowing them to pair with different amplified cargoes. This modularity could allow manufacturers to bypass cost- and time-intensive optimization experiments required to optimize the conditions with individual saRNAs.

5 Applications of RNA Therapeutics

In 1990, it was demonstrated that naked exogenous mRNA injected into murine skeletal muscle can translate into a protein at the injection site (Wolff et al. 1990). In 1992, proof-of-principal for using mRNA as a therapeutic has provided. In this case, intracranial injection of mRNA encoding vasopressin in a rodent model of diabetes insipidus temporally reversed the disease (Jirikowski et al. 1992). In 1995, the possibility therapeutic utility of vaccination with mRNA encoding a carcinoembryonic antigen was demonstrated (Conry et al. 1995).

The vaccines against SARS-CoV-2 became the first RNA-based drugs approved by FDA with emergency use authorization in 2020 (Biointech/Pfizer's BNT162b2; and Moderna's mRNA-1273) followed by full approval in 2021. Both vaccines encode membrane-anchored full-length spike protein of the viral capsid. Two proline mutations were introduced into the sequence to increase protein stability. The spike RNA is encapsulated into lipid nanoparticles and delivered intramuscularly in two doses several weeks apart. Data obtained in phase III trials suggest that these vaccines are safe and effective: 95% efficacy for BNT162b2 and 94.1% efficacy for mRNA-1273 for the current SARS-CoV-2 strains (Polack et al. 2020; Baden et al. 2021) (at the time of this writing, the efficacy of the vaccines against the Omicron variant is unknown). It is worth noting that these vaccines were developed with unprecedented speed—for instance, mRNA-1273 was generated and administered to the first study participant just 63 days after the SARS-CoV-2 genome sequence was published (Moderna 2020). Currently, there are many preventive mRNA vaccines under development, including those aimed at protecting from rabies (Aldrich et al. 2021), cytomegalovirus infection (ModernaTX Inc. 2021a), HIV (International AIDS Vaccine Initiative 2020), Zika virus (ModernaTX, Inc. 2021b), influenza virus (Bahl et al. 2017), and others. In addition to a rapid development time, RNA vaccines have some other advantageous characteristics, such as being able to deliver an encoded antigen in an HLA-independent manner and to act as natural adjuvants due to their TLR7/8 ligand activity (Grunwitz et al. 2019).

RNA technology can also be used to make therapeutic cancer vaccines. In this case, RNA encodes tumor neoantigens, shared antigens or combination of both. For instance, CV9202 is a vaccine encoding six shared antigens commonly found in non-small cell lung cancer (Papachristofilou et al. 2019). mRNA-5671 is a vaccine against KRAS-positive cancers, such as pancreatic or colon cancer (Merck Sharp & Dohme Corp. 2020). The vaccine against HPV16 is another promising example of therapeutic RNA vaccines—it encodes E6 and E7 oncoproteins of HPV and is administered systemically to induce potent and durable CD8^{+} T cell response (Grunwitz et al. 2019). Of note, the encoded antigens in this vaccine are fused to the MHC class I signal sequence and transmembrane and cytoplasmic domains for routing to the endoplasmic reticulum, resulting in increased presentation efficacy of MHC class I and II epitopes (Kreiter et al. 2008). Such optimization of vaccine design reflects the flexibility of RNA technology and is often implemented to increase antitumor efficacy. A vaccine encoding four shared melanoma-associated antigens, for instance, was enhanced by incorporating a RIG-I immunostimulatory sequence (Heidegger et al. 2019). Examples of personalized vaccines include mRNA-4157 and BNT122, which constructs neoantigens identified through genetic sequencing and bioinformatic analysis of a patient's tumor (van Dülmen and Rentmeister 2020; Jou et al. 2021).

Recently, the proof-of-concept for using RNA as tolerogenic vaccine was provided. In contrast to a vaccine augmenting immune responses against a pathogen, a tolerogenic vaccine is aimed at inducing tolerogenic responses to suppress or impair immunity against antigens, which in most cases are auto-antigens or allergens (Geng et al. 2015). Such vaccines may help in managing autoimmune diseases. Indeed, an RNA encoding a multiple sclerosis-related antigen suppressed the progression of this autoimmune disease in several mouse models. The treatment effect was associated with a reduction of effector T cells and the development of regulatory T cell populations, which executed strong bystander immunosuppression and thus improved disease induced by cognate and noncognate auto-antigens (Krienke et al. 2021).

RNA can also be used to enhance dendritic cell (DC)-mediated immunotherapy for cancer. In this approach, mRNA is delivered into patient's DCs ex vivo or in vivo to induce their maturation and enhance their ability to stimulate T cells. For example, TriMix is a cocktail of RNA encoding CD40L, CD70, and constitutively active toll-like-receptor 4 (caTLR4) that is transfected into DCs ex vivo. CD40L and caTLR4 promote maturation of cytokine-secreting DCs, whereas CD70 enhances T cell proliferation. When TriMix cocktail was co-transfected with melanoma antigens and co-cultured with autologous naïve CD8 + T cells, a 500-fold increase in antigen-specific CD8 + T cells was observed when compared with immature DCs, and a 200-fold increase when compared with cytokine cocktail-matured DCs (Bonehill et al. 2008). TriMix-based therapeutic vaccine, TriMixDC-MEL, in combination with ipilimumab, a cytotoxic T-lymphocyte-associated protein 4 blocker, induced an encouraging rate of highly durable tumor responses in patients with advanced melanoma (Wilgenhof et al. 2016).

RNA encoding chimeric antigen receptors (CAR) for T cells is another promising avenue for RNA technology. CAR can be designed to recognize any desired antigen on the cell surface so that cytotoxic T cell expressing the CAR will detect and destroy other cells bearing that antigen. Usually, T cells are expanded and transfected with CAR-encoding RNA ex vivo, then infused back into the patient (Krug et al. 2014; Foster et al. 2019). Descartes-08 is an RNA-based CAR T cell therapy to treat myasthenia gravis. In this autoimmune disease, pathogenic autoantibodies produced by plasma cells bind to and destroy neuromuscular synapses resulting in muscle weakness and rapid fatigue. Plasma cell clones producing autoantibodies also express B cell maturation antigen (BCMA). Descartes-08 can recognize and eliminate BCMA-expressing cells and is now in Phase 1/2 clinical trials (Cartesian Therapeutics 2021a; Cartesian). Similarly, Descartes-11 eliminates BCMA-expressing myeloma cells and is in development to treat newly diagnosed patients with high-risk multiple myeloma (Cartesian Therapeutics 2021b; Cartesian 2021). Recently, it has been demonstrated that CAR RNA can be delivered into T cells in vivo with lipid nanoparticles carrying anti-CD3 antibodies. Such particles accumulated in the organs enriched in T cells, such as spleen, thymus, and lymph nodes, and, when loaded with CAR RNA, could reprogram host T cells to recognize and eliminate leukemia cells and prostate cancer cells in animal models, prolonging survival significantly (Parayath et al. 2020).

RNA is also an attractive technology for genome editing as it can ensure transient expression of genome editing tools. Several preclinical and clinical studies using RNA encoding zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated protein 9 (Cas9) are ongoing (Zhang et al. 2019). For example, SB-728mR-T encodes ZFN targeting chemokine receptor (CCR5) gene. The latter is a surface protein present on the white blood cells which serves as a co-receptor for HIV virus and certain mutations of this receptor grant natural resistance to the virus. SB-728mR-T is designed to delete 32 bp in CCR5 gene making the recipient resistant to HIV. In currently ongoing clinical study, the drug is electroporated ex vivo into autologous hematopoietic stem/progenitor cells of HIV-1 infected patients preconditioned with busulfan (DiGiusto et al. 2016; City of Hope Medical Center 2020). Another RNA-based gene-editing drug, NTLA-2001, is targeted against transthyretin amyloidosis. This RNA construct encodes a CRISPR-Cas9 system, encapsulated into nanoparticles formulated to achieve preferential delivery to the liver. NTLA-2001 knocks out the hepatic TTR gene responsible for the production of misfolded transthyretin protein. A single dose of NTLA-2001 edits more than 70% of the genome in the liver of non-human primates and reduces serum transthyretin concentration more than 80% in patients with amyloidosis (Intellia Therapeutics 2020; Gillmore et al. 2021). Another application is the use of an mRNA CRISPR-Cas9 system, delivered in lipid nanoparticles, to knock out hepatic PCSK9 gene and thereby reduce LDL cholesterol levels in non-human primates. PCSK9 is a negative regulator of LDL receptors (LDLR) which bind to and clear LDL cholesterol from circulation (Musunuru et al. 2021). Both LDLR and PCSK9 are highly expressed in the liver, thereby making this organ the main target for RNA-based gene therapy of hypercholesterolemia.

Since RNA can encode any protein, the number of therapeutic applications for RNA technology is indeed countless and examples above represent only a small portion of RNA-based therapeutics under development (Damase et al. 2021). Gene replacement therapy with RNA can reverse a deficiency of key metabolic enzymes causing a disease process. For instance, ARCT-810, now in early clinical trials, is designed to supply a functional transcript of ornithine transcarbamylase (OTC) (Arcturus Therapeutics, Inc. 2020, 2021). This enzyme helps to eliminate ammonia through urea cycle in the liver. Genetic defects causing a deficiency of OTC result in hyperammonemia and subsequent neurological derangements. Promising preclinical data have also been reported for RNA-based replacement therapy in the models of α-1-antitrypsin deficiency (Connolly et al. 2018), citrin deficiency (Cao et al. 2019), lysosomal storage diseases (Zhu et al. 2019), and glycogen storage diseases (Cao et al. 2021).

In addition, RNA-enhanced cell therapies and bioengineering are on the horizon. As one example, we have shown that mRNA encoding human telomerase can extend telomeres in senescent human cells, increase replicative capacity, reverse the senescence-associated secretory phenotype, reduce DNA damage, and restore normal cellular functions (Ramunas et al. 2015; Li et al. 2017, 2019; Mojiri et al. 2021). This promising work has stimulated preclinical studies to assess the benefit of this mRNA therapy to enhance an FDA-approved cell therapy product for burn patients.

6 Conclusion and Future Perspective

The future is bright for the field of mRNA therapeutics. Current mRNA technology provides a platform to rapidly develop and deploy vaccines against infectious diseases; to personalize therapies for cancer and genetic diseases; to generate therapeutic proteins to address previously "undruggable" targets; to address the major illnesses that afflict modern society including cardiovascular and neurological disorders. Our hospital-based program is designed to help small companies and academic groups with a great RNA idea get to the clinic. Such programs will increase the number and the diversity of RNA solutions for disease. In addition, hospital-based programs will address current gaps in medical care for orphan diseases and will provide personalized RNA therapies for their patients. Advances in tissue targeting and in the stability and translational efficiency of mRNA will increase its application for a broad range of diseases. The opportunities for RNA therapeutics are almost limitless, as we stand on the threshold of a brave new world.

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References

Aldrich C, Leroux-Roels I, Huang KB et al (2021) Proof-of-concept of a low-dose unmodified mRNA-based rabies vaccine formulated with lipid nanoparticles in human volunteers: a phase 1 trial. Vaccine 39:1310–1318

Alexopoulou L, Holt AC, Medzhitov R et al (2001) Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413:732–738

Arcturus Therapeutics, Inc. (2020) A phase 1 randomized, double blinded, placebo controlled, ascending dose study to assess the safety, tolerability, and pharmacokinetics of single doses of ARCT-810 in healthy adult subjects. clinicaltrials.gov

Arcturus Therapeutics, Inc. (2021) A phase 1b randomized, double blinded, placebo controlled, ascending dose study to assess the safety, tolerability, and pharmacokinetics of single doses of ARCT-810 in clinically stable patients with ornithine transcarbamylase deficiency. clinicaltrials.gov

Baden LR, El Sahly HM, Essink B et al (2021) Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med 384:403–416

Bahl K, Senn JJ, Yuzhakov O et al (2017) Preclinical and clinical demonstration of immunogenicity by mRNA vaccines against H10N8 and H7N9 influenza viruses. Mol Ther 25:1316–1327

Beaudry D, Perreault JP (1995) An efficient strategy for the synthesis of circular RNA molecules. Nucleic Acids Res 23:3064–3066

Blakney AK, Ip S, Geall AJ (2021) An Update on self-amplifying mRNA vaccine development. Vaccines (basel) 9:97

Bloom K, van den Berg F, Arbuthnot P (2021) Self-amplifying RNA vaccines for infectious diseases. Gene Ther 28:117–129

Boada C, Zinger A, Tsao C et al (2020) Rapamycin-loaded biomimetic nanoparticles reverse vascular inflammation. Circ Res 126:25–37

Bogers WM, Oostermeijer H, Mooij P et al (2015) Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV type 1 envelope with a cationic nanoemulsion. J Infect Dis 211:947–955

Bonehill A, Tuyaerts S, Van Nuffel AM et al (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Mol Ther 16:1170–1180

Caccomo S (2019) FDA approves innovative gene therapy to treat pediatric patients with spinal muscular atrophy, a rare disease and leading genetic cause of infant mortality. In: FDA. https://www.fda.gov/news-events/press-announcements/fda-approves-innovative-gene-therapy-treat-pediatric-patients-spinal-muscular-atrophy-rare-disease. Accessed 30 Jul 2021

Cao J, An D, Galduroz M et al (2019) mRNA therapy improves metabolic and behavioral abnormalities in a murine model of citrin deficiency. Mol Ther 27:1242–1251

Cao J, Choi M, Guadagnin E et al (2021) mRNA therapy restores euglycemia and prevents liver tumors in murine model of glycogen storage disease. Nat Commun 12:3090

Cartesian cartesian clinical trials. In: cartesian. http://www.cartesiantherapeutics.com/clinical-trials/. Accessed 9 Aug 2021

Cartesian (2021) Cartesian therapeutics initiates phase 2 clinical trial of first RNA-engineered cell therapy for frontline cancer. In: Cartesian. http://www.cartesiantherapeutics.com/cartesian-therapeutics-initiates-phase-2-clinical-trial-of-first-rna-engineered-cell-therapy-for-frontline-cancer/. Accessed 9 Aug 2021

Cartesian Therapeutics (2021a) autologous T-cells expressing a chimeric antigen receptor directed to B-Cell maturation antigen (BCMA) In: Patients with generalized myasthenia gravis (MG). clinicaltrials.gov

Cartesian Therapeutics (2021b) descartes-11 consolidation treatment in patients with high-risk multiple myeloma who have residual disease after induction therapy. clinicaltrials.gov

Cheng Q, Wei T, Farbiak L et al (2020) Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing. Nat Nanotechnol 15:313–320

City of Hope Medical Center (2020) A pilot study to evaluate the feasibility, safety and engraftment of zinc finger nuclease (ZFN) CCR5 modified CD34+ hematopoietic stem/progenitor cells (SB-728mR-HSPC) in HIV-1 (R5) infected patients. clinicaltrials.gov

Collett MS, Faras AJ (1976) Evidence for circularization of the avian oncornavirus RNA genome during proviral DNA synthesis from studies of reverse transcription in vitro. Proc Natl Acad Sci USA 73:1329–1332

Connolly B, Isaacs C, Cheng L et al (2018) SERPINA1 mRNA as a treatment for alpha-1 antitrypsin deficiency. J Nucleic Acids 2018:8247935

Conry RM, LoBuglio AF, Wright M et al (1995) Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res 55:1397–1400

Corbett KS, Gagne M, Wagner DA et al (2021) Protection against SARS-CoV-2 beta variant in mRNA-1273 vaccine-boosted nonhuman primates. Science 374:1343–1353

Crommelin DJA, Anchordoquy TJ, Volkin DB et al (2021) Addressing the cold reality of mRNA vaccine stability. J Pharm Sci 110:997–1001

Damase TR, Sukhovershin R, Boada C et al (2021) The limitless future of RNA therapeutics. Front Bioeng Biotechnol 9:628137

DiGiusto DL, Cannon PM, Holmes MC et al (2016) Preclinical development and qualification of ZFN-mediated CCR5 disruption in human hematopoietic stem/progenitor cells. Mol Ther Methods Clin Dev 3:16067

Ford E, Ares M (1994) Synthesis of circular RNA in bacteria and yeast using RNA cyclase ribozymes derived from a group I intron of phage T4. Proc Natl Acad Sci USA 91:3117–3121

Foster JB, Barrett DM, Karikó K (2019) The emerging role of in vitro-transcribed mRNA in adoptive T cell immunotherapy. Mol Ther 27:747–756

Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci USA 109:14604–14609

Geng S, Zhang H, Zhou X et al (2015) Diabetes tolerogenic vaccines targeting antigen-specific inflammation. Hum Vaccin Immunother 11:522–530

Gillmore JD, Gane E, Taubel J et al (2021) CRISPR-cas9 in vivo gene editing for transthyretin amyloidosis. N Engl J Med 385:493–502

Green AA, Silver PA, Collins JJ et al (2014) Toehold switches: de-novo-designed regulators of gene expression. Cell 159:925–939

Green MR, Sambrook J (2019) Removing DNA contamination from RNA samples by treatment with RNase-free DNase I. Cold Spring Harb Protoc 2019:pdb.prot101725

Green MR, Sambrook J (2021) Separation of RNA according to Size: Electrophoresis of RNA through Denaturing Urea Polyacrylamide Gels. Cold Spring Harb Protoc 2021:pdb.prot101766

Grunwitz C, Salomon N, Vascotto F et al (2019) HPV16 RNA-LPX vaccine mediates complete regression of aggressively growing HPV-positive mouse tumors and establishes protective T cell memory. Oncoimmunology 8:e1629259

Hanewich-Hollatz MH, Chen Z, Hochrein LM et al (2019) Conditional guide RNAs: programmable conditional regulation of CRISPR/Cas function in bacterial and mammalian cells via dynamic RNA nanotechnology. ACS Cent Sci 5:1241–1249

Heidegger S, Kreppel D, Bscheider M et al (2019) RIG-I activating immunostimulatory RNA boosts the efficacy of anticancer vaccines and synergizes with immune checkpoint blockade. EBioMedicine 41:146–155

Heil F, Hemmi H, Hochrein H et al (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526–1529

Henderson JM, Ujita A, Hill E et al (2021) Cap 1 messenger RNA synthesis with co-transcriptional CleanCap® analog by in vitro transcription. Curr Protoc 1:e39

Hewitt SL, Bailey D, Zielinski J et al (2020) Intratumoral IL12 mRNA therapy promotes TH1 transformation of the tumor Microenvironment. Clin Cancer Res 26:6284–6298

Hornung V, Ellegast J, Kim S et al (2006) 5′-triphosphate RNA is the ligand for RIG-I. Science 314:994–997

Intellia Therapeutics (2020) Phase 1 two-part (open-label, single ascending dose (part 1) and open-label, single dose expansion (part 2)) study to evaluate safety, tolerability, pharmacokinetics, and pharmacodynamics of NTLA-2001 in patients with hereditary transthyretin amyloidosis with polyneuropathy (ATTRv-PN). clinicaltrials.gov

International AIDS Vaccine Initiative (2020) A Phase 1, randomized, double-blind, placebo-controlled dosage escalation trial to evaluate the safety and immunogenicity of eOD-GT8 60mer Vaccine, Adjuvanted in HIV-uninfected, Healthy Adult Volunteers. clinicaltrials.gov

Jackson LA, Anderson EJ, Rouphael NG et al (2020) An mRNA vaccine against SARS-CoV-2—preliminary report. N Engl J Med 383:1920–1931

Jirikowski GF, Sanna PP, Maciejewski-Lenoir D et al (1992) Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA. Science 255:996–998

Jou J, Harrington KJ, Zocca M-B et al (2021) The changing landscape of therapeutic cancer vaccines—novel platforms and neoantigen identification. Clin Cancer Res 27:689–703

Kahn J (2019) FDA grants accelerated approval to first targeted treatment for rare Duchenne muscular dystrophy mutation. In: FDA. https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-first-targeted-treatment-rare-duchenne-muscular-dystrophy-mutation. Accessed 30 Jul 2021

Karikó K, Buckstein M, Ni H et al (2005) Suppression of RNA recognition by toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23:165–175

Karikó K, Muramatsu H, Ludwig J et al (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res 39:e142

Karikó K, Muramatsu H, Welsh FA et al (2008) Incorporation of pseudouridine Into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther 16:1833–1840

Kim J, Hu C, Moufawad El Achkar C et al (2019) Patient-customized oligonucleotide therapy for a rare genetic disease. N Engl J Med 381:1644–1652

Kormann MSD, Hasenpusch G, Aneja MK et al (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat Biotechnol 29:154–157

Kozak M (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J Mol Biol 196:947–950

Kreiter S, Selmi A, Diken M et al (2008) Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol 180:309–318

Krienke C, Kolb L, Diken E et al (2021) A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. Science 371:145–153

Krug C, Wiesinger M, Abken H et al (2014) A GMP-compliant protocol to expand and transfect cancer patient T cells with mRNA encoding a tumor-specific chimeric antigen receptor. Cancer Immunol Immunother 63:999–1008

Li Y, Zhou G, Bruno IG et al (2019) Transient introduction of human telomerase mRNA improves hallmarks of progeria cells. Aging Cell 18:e12979

Li Y, Zhou G, Bruno IG et al (2017) Telomerase mRNA reverses senescence in progeria cells. J Am Coll Cardiol 70:804–805

MacFadden A, O'Donoghue Z, Silva PAGC et al (2018) Mechanism and structural diversity of exoribonuclease-resistant RNA structures in flaviviral RNAs. Nat Commun 9:119
Magadum A, Kurian AA, Chepurko E et al (2020) Specific modified mRNA translation system. Circulation 142:2485–2488
Mamcarz E, Zhou S, Lockey T et al (2019) Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. N Engl J Med 380:1525–1534
Martin S, Moss B (1975) Modification of RNA by mRNA guanylyltransferase and mRNA (guanine-7-)methyltransferase from vaccinia virions. J Biol Chem 250:9330–9335
Mauger DM, Cabral BJ, Presnyak V et al (2019) mRNA structure regulates protein expression through changes in functional half-life. Proc Natl Acad Sci USA 116:24075–24083
McKenna SA, Kim I, Puglisi EV et al (2007) Purification and characterization of transcribed RNAs using gel filtration chromatography. Nat Protoc 2:3270–3277
Merck Sharp & Dohme Corp. (2020) A Phase 1, open-label, multicenter study to assess the safety and tolerability of mRNA-5671/V941 as a monotherapy and in combination with pembrolizumab in participants with KRAS mutant advanced or metastatic non-small cell lung cancer, colorectal cancer or pancreatic adenocarcinoma. clinicaltrials.gov
Moderna I (2020) Moderna announces first participant dosed in NIH-led phase 1 study of mRNA vaccine (mRNA-1273) against novel coronavirus. https://investors.modernatx.com/news/news-details/2020/Moderna-Announces-First-Participant-Dosed-in-NIH-led-Phase-1-Study-of-mRNA-Vaccine-mRNA-1273-Against-Novel-Coronavirus-03-16-2020/default.aspx. Accessed 8 Dec 2021
ModernaTX, Inc. (2021a) A phase 2, randomized, observer-blind, placebo-controlled, dose-finding trial to evaluate the safety and immunogenicity of cytomegalovirus vaccine mRNA-1647 in healthy adults. clinicaltrials.gov
ModernaTX, Inc. (2021b) A Phase 2, randomized, observer-blind, placebo-controlled, dose confirmation study to evaluate the safety, tolerability, and immunogenicity of zika vaccine mRNA-1893 in adults aged 18 through 65 years and living in endemic and non-endemic Flavivirus areas. clinicaltrials.gov
Mojiri A, Walther BK, Jiang C et al (2021) Telomerase therapy reverses vascular senescence and extends lifespan in progeria mice. Eur Heart J 42:4352–4369
Molinaro R, Pasto A, Taraballi F et al (2020) Biomimetic nanoparticles potentiate the anti-inflammatory properties of dexamethasone and reduce the cytokine storm syndrome: an additional weapon against COVID-19? Nanomaterials (basel) 10:2301
Musunuru K, Chadwick AC, Mizoguchi T et al (2021) In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. Nature 593:429–434
Papachristofilou A, Hipp MM, Klinkhardt U et al (2019) Phase Ib evaluation of a self-adjuvanted protamine formulated mRNA-based active cancer immunotherapy, BI1361849 (CV9202), combined with local radiation treatment in patients with stage IV non-small cell lung cancer. J Immunother Cancer 7:38
Parayath NN, Stephan SB, Koehne AL et al (2020) In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo. Nat Commun 11:6080
Pardi N, Muramatsu H, Weissman D et al (2013) In Vitro transcription of long RNA containing modified nucleosides. In: Rabinovich PM (ed) Synthetic messenger RNA and Cell metabolism modulation. Humana Press, Totowa, NJ, pp 29–42
Parr CJC, Wada S, Kotake K et al (2020) N1-Methylpseudouridine substitution enhances the performance of synthetic mRNA switches in cells. Nucleic Acids Res 48:e35
Pijlman GP, Funk A, Kondratieva N et al (2008) A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. Cell Host Microbe 4:579–591
Polack FP, Thomas SJ, Kitchin N et al (2020) Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. N Engl J Med 383:2603–2615
Puttaraju M, Been MD (1992) Group I permuted intron-exon (PIE) sequences self-splice to produce circular exons. Nucleic Acids Res 20:5357–5364

Ramunas J, Yakubov E, Brady JJ et al (2015) Transient delivery of modified mRNA encoding TERT rapidly extends telomeres in human cells. FASEB J 29:1930–1939

Scaglioni D, Catapano F, Ellis M et al (2021) The administration of antisense oligonucleotide golodirsen reduces pathological regeneratio in patients with Duchenne muscular dystrophy. Acta Neuropathol Commun 9:7

Summer H, Grämer R, Dröge P (2009) Denaturing urea polyacrylamide gel electrophoresis (Urea PAGE). J Vis Exp 1485

van Dülmen M, Rentmeister A (2020) mRNA Therapies: new hope in the fight against melanoma. Biochemistry 59:1650–1655

Waldrop MA, Karingada C, Storey MA et al (2020) Gene therapy for spinal muscular atrophy: safety and early outcomes. Pediatrics 146

Wesselhoeft RA, Kowalski PS, Anderson DG (2018) Engineering circular RNA for potent and stable translation in eukaryotic cells. Nat Commun 9:2629

Wesselhoeft RA, Kowalski PS, Parker-Hale FC et al (2019) RNA circularization diminishes immunogenicity and can extend translation duration in vivo. Mol Cell 74:508-520.e4

Wilgenhof S, Corthals J, Heirman C et al (2016) Phase II study of autologous monocyte-derived mRNA electroporated dendritic cells (TriMixDC-MEL) plus ipilimumab in patients with pretreated advanced melanoma. J Clin Oncol 34:1330–1338

Wolff JA, Malone RW, Williams P et al (1990) Direct gene transfer into mouse muscle in vivo. Science 247:1465–1468

Yang W (2011) Nucleases: diversity of structure, function and mechanism. Q Rev Biophys 44:1–93

Yoneyama M, Kikuchi M, Matsumoto K et al (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 175:2851–2858

Yoneyama M, Kikuchi M, Natsukawa T et al (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737

Zhang H-X, Zhang Y, Yin H (2019) Genome editing with mRNA encoding ZFN, TALEN, and Cas9. Mol Ther 27:735–746

Zhao EM, Mao AS, de Puig H et al (2021) RNA-responsive elements for eukaryotic translational control. Nat Biotechnol 1–7

Zhao M, Woodside MT (2021) Mechanical strength of RNA knot in Zika virus protects against cellular defenses. Nat Chem Biol 17:975–981

Zhu X, Yin L, Theisen M et al (2019) Systemic mRNA therapy for the treatment of fabry disease: preclinical studies in wild-type mice, fabry mouse model, and wild-type non-human primates. Am J Hum Genet 104:625–637