October 2019

Design and Delivery of Synthetic mRNA by a Peptide Nanoparticle

John H. Lockhart

University of South Florida

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Design and Delivery of Synthetic mRNA by a Peptide Nanoparticle

by

John H. Lockhart

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Molecular Pharmacology and Physiology Department of Molecular Pharmacology and Physiology College of Medicine University of South Florida

Major Professor: Hana Totary-Jain, Ph.D. Hua Pan, Ph.D. Jerome Breslin, Ph.D. Thomas Taylor-Clark, Ph.D.

Date of Approval: August 23, 2019

Keywords: microRNA switch, nucleotide modification, endosome, atherosclerosis

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Dedication

For my wife Amber. The world is a brighter place because of you, and I hope that we get to see it all.
Acknowledgements

I would like to thank my major professor, Dr. Hana Totary-Jain for her mentorship, guidance, and support throughout my doctoral studies at USF. Her enthusiasm and passion for research is something that I have aspired to match since my first day in her lab. I am certain that the training and opportunities for growth that she has provided to me will be invaluable throughout my future endeavors.

I would also like to thank the members of my dissertation committee: Dr. Hua Pan, Dr. Jerome Breslin and Dr. Thomas Taylor-Clark. Their readiness to answer questions, offer technical advice, and even provide reagents has been invaluable to me.

Thank you to the former members of the Totary-Jain lab, Dr. John Canfield, Dr. Ezinne Mong, for their support throughout my time in the lab and providing me with opportunities to assist in their own research.

I must give a special “thank you” to Jeffrey VanWye, without whom this work would have taken at least twice as long and because of whom was twice as enjoyable.
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<td>4E-BP</td>
<td>eIF4E-Binding Protein</td>
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<td>A-Site</td>
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<tr>
<td>aa-tRNA</td>
<td>Aminoacyl-Transfer RNA</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<td>ABCE</td>
<td>ATP Binding Cassette Subfamily E</td>
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<td>Ad-HEK293</td>
<td>Adherent-Human Embryonic Kidney cells</td>
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<td>Argonaute</td>
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<td>ARCA</td>
<td>Anti-Reverse Cap Analog</td>
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<td>ARE</td>
<td>AU-rich Element</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>Codon Adaptation Index</td>
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<td>CCV</td>
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<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
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<td>Definition</td>
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<td>Double-Stranded RNA</td>
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<td>General Control Nonderepressible 2</td>
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<td>GRE</td>
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<td>HPLC</td>
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<td>HRI</td>
<td>Hemin-Regulated Inhibitor Kinase</td>
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<td>Hsp</td>
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<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
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<td>Abbreviation</td>
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<td>Interferon</td>
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<td>IκB kinase</td>
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<td>Interleukin 12</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<td>iPSC</td>
<td>Induced Pluripotent Stem Cells</td>
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<td>Inhibitor κB</td>
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<td>MPS</td>
<td>Mononuclear Phagocyte System</td>
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<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<td>MYD88</td>
<td>Myeloid Differentiation Primary Response 88</td>
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<td>NF-κB</td>
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<td>niRFP</td>
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<td>Non-Structural Protein</td>
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<td>Polymerase Chain Reaction</td>
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<td>Polyethylene Glycol</td>
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<td>PEI</td>
<td>Polyethylenimine</td>
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<td>PELO</td>
<td>Pelota mRNA Surveillance and Ribosome Rescue Factor</td>
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<td>PERK</td>
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<td>Propidium Iodide</td>
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<td>Poly(A) tail</td>
<td>Polyadenylate Tail</td>
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<td>pre-mRNA</td>
<td>Precursor mRNA</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PRF</td>
<td>Programmed Ribosomal Frameshifting</td>
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<td>Primary microRNA</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
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<td>RIG-I</td>
<td>Retinoic Acid Inducible Gene-I</td>
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<tr>
<td>RISC</td>
<td>RNA-induced Silencing Complex</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<td>2'-5' Oligoadenylate Synthetase-dependent Ribonuclease</td>
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<td>siRNA</td>
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<td>SMG</td>
<td>Suppressor with Morphological Effect on Genitalia</td>
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<td>Single-Stranded RNA</td>
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<td>SURF</td>
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<td>Tumor Necrosis Factor Alpha</td>
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<td>TNF Receptor-associated Factor</td>
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<td>TRBP</td>
<td>TAR-RNA Binding Protein</td>
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<td>Description</td>
</tr>
<tr>
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<tr>
<td>TRIF</td>
<td>TIR-Domain-containing Adapter-inducing Interferon-β</td>
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<td>Transfer RNA</td>
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<td>tRNAiMet</td>
<td>Methionine Initiator Transfer RNA</td>
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<tr>
<td>UPF</td>
<td>Regulator of Nonsense Mediated Decay</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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<tr>
<td>vATPase</td>
<td>Vacuolar-type H+-ATPase</td>
</tr>
<tr>
<td>VEE</td>
<td>Venezuelan Equine Encephalitis Virus</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cells</td>
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<tr>
<td>ZFN</td>
<td>Zinc-Finger Nuclease</td>
</tr>
<tr>
<td>Ψ</td>
<td>Pseudouridine</td>
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Abstract

The field of synthetic mRNA therapeutics is a rapidly expanding arm of gene therapies. The use of mRNA provides multiple benefits over viral or DNA vectors. Synthetic mRNA vectors are immediately translated into protein after entering the cytoplasm of cells in contrast to DNA vectors that must first be transcribed to mRNA in the nucleus. This allows synthetic mRNA to produce a therapeutic protein in any cell type, including non-dividing cells. In addition, the non-replicative nature of mRNA means that insertional mutagenesis or generation of escape mutants is not a concern. However, the stimulation of innate immune responses by unmodified synthetic mRNA prevented widespread clinical applications.

The discovery that incorporation of modified nucleotides, such as pseudouridine or 5-methylcytosine, prevents the recognition by innate immune sensors has renewed interest in the use of synthetic mRNA as a therapeutic. In conjunction, numerous post-transcriptional regulatory elements have recently been described in mRNA. Adding these regulatory elements to synthetic mRNA allows control of the expression of the encoded protein in tissue-, cell-, or environmental-specific conditions. However, the influence that the modified nucleotides commonly incorporated in synthetic mRNA have on the regulatory capacity of these elements has not been examined.

In this study we investigated what effects modified nucleotides have on the regulation of synthetic mRNA by microRNA (miRNA switch). We found that nucleotide
modifications that increase the translation of the synthetic mRNA tended to decrease
the regulatory capacity of microRNA switch. Inclusion of multiple microRNA target sites
at the 3’ UTR of the synthetic mRNA was able to minimize the loss of miRNA-
dependent regulation of the miRNA switch, but microRNA target sites complementary to
the six-nucleotide microRNA “seed” sequence were more affected to nucleotide
modification. We found the effect of nucleotide modifications varied between microRNA
species and was not determined by the proportion of modified nucleotides present in the
microRNA target sites. Finally, we observed that utilizing a single microRNA target site
at the 5’ UTR of the synthetic mRNA completely ameliorated the loss of regulation due
to nucleotide modifications.

Because synthetic mRNA are easy to produce and can be made to encode any
protein of interest, they are ideal for clinical development. Currently there are over 45
clinical trials underway utilizing synthetic mRNA as a monotherapy or in conjunction with
other therapeutics. Most of these clinical trials are focused on cancer immunotherapy,
particularly autologous T-cell therapy. This therapeutic modality is well suited for
synthetic mRNA as the target cells are transfected ex vivo. This avoids the major
obstacles that synthetic mRNA therapeutics must still overcome: delivery to target
organs.

The sensitivity of synthetic mRNA to extracellular ribonucleases requires
encapsulation of the mRNA in a protective nanoparticle. Numerous such nanoparticles
have been reported, but nearly all are variations on either lipid nanoparticles or
polymeric nanoparticles. The advances made thus far with these two mRNA delivery
platforms have significantly reduced their toxicity, however the endosomal escape rate
of these particles remains well below 5%. Furthermore, when administered systemically these nanoparticles are avidly taken up by sentinel macrophages of the liver and spleen or hepatocytes. The accumulation of particles in the liver has thus far limited the applications of mRNA therapeutics to diseases and disorders that are liver-specific or that can be treated by using the liver as a biosynthetic depot. Expanding the clinical application of synthetic mRNA may require the discovery of novel delivery platforms that are capable of targeting other organs.

In this study we also tested the delivery of synthetic mRNA using a small cell penetrating peptide, called p5RHH, that is derived from bee venom protein melittin. We showed that in the presence of mRNA, p5RHH self-assembles into spherical nanoparticles that display a high degree of RNase resistance. These nanoparticles were consistently sized regardless of the length of the mRNA payload. Furthermore, after uptake by cells, p5RHH-mRNA nanoparticles displayed a high degree of endosomal escape that was dependent upon the acidification of endosomes, which disassembles the nanoparticles. The high concentration of p5RHH in the lumen of the endosome led to efficient endosomal disruption and produces minimal cytotoxic effects. When the p5RHH-mRNA nanoparticles were injected intravenously into an atherosclerotic mouse, we observed robust expression of the payload mRNA in only the atherosclerotic plaques. The lack of expression in typical depot organs, such as the liver, spleen, lungs, or kidneys, was also confirmed in a normal mouse. The simplicity and specificity of p5RHH-mRNA nanoparticles makes them an ideal candidate for further pre-clinical development as an mRNA delivery platform.
mRNA Biogenesis

The expression of mRNA is the primary phenotypic determinant of a cell; the epigenetic patterning of a cell’s DNA gives rise to a pool of mRNAs that are dynamically regulated by their protein products in response to internal and external stimuli. The production of eukaryotic mRNA is a highly regulated and concerted process, that includes transcription from genomic DNA and processing of transcripts to form mature mRNA.

Transcription of pre-mRNA

The production of mRNA begins with the transcription of precursor (pre)-mRNA from DNA by RNA polymerase II. RNA polymerase II is recruited to the promoter region of a gene by DNA-bound transcription factors. Because the recruitment of RNA polymerase II to the promoter does not displace the transcription factors bound there, multiple polymerases can transcribe the same gene simultaneously 1.

Once bound, the RNA polymerase II unwinds the double-stranded DNA and begins to synthesize an RNA version of the coding strand of DNA based on Watson-Crick base pairing of free ribonucleotides with the complementary strand of DNA. The pre-mRNA transcript is elongated as the polymerase continues along the gene. Termination of transcription by RNA polymerase II is caused by recognition of the
polyadenylation signal which leads to cleavage and release of the nascent pre-mRNA\(^2\). Importantly, RNA polymerase II contains an unstructured C-terminal domain that regulates its activity and also localizes proteins necessary for processing the nascent transcript\(^2\).

**Addition of the 5' Cap**

The first step of processing of pre-mRNA into mature mRNA is the addition of the 5’ cap. Capping of mRNA is essential in eukaryotes for avoidance of the innate immune system and recognition of mRNA by conventional translation initiators. Though, under stress conditions cap-independent translation can be used to express a specific subset of the cellular mRNA pool\(^3\). Capping begins after approximately 25 nucleotides have been transcribed. The initial enzymatic step by RNA triphosphatase converts the 5’ triphosphate of the nascent pre-mRNA into a diphosphate. This allows RNA guanyltransferase to attach a guanosine monophosphate to the 5’ of the pre-mRNA to form a 5’-5’ triphosphate bond. The attached guanosine is then methylated at N7 to produce the 7-methylguanosine (m7G) Cap0. In higher order eukaryotes, the Cap0 is further modified by the addition of another methyl group to the 2’ hydroxyl of the ribose sugar of the neighboring nucleotide to produce Cap1\(^4\). Additional methylation of 2’ hydroxyl of the second transcribed nucleotide to form Cap2 occurs in about half of all mRNAs, which unlike Cap1 can also occur in the cytoplasm after nuclear export of the mature mRNA.
**Splicing of pre-mRNA**

In eukaryotes, the coding portions of a gene may be interspersed with non-coding regions, called exons and introns respectively. Transcription of a gene includes both of these regions. However, the intronic regions of a gene are rapidly spliced out of the transcript by the spliceosome. The assembly and function of the human spliceosome involves approximately 300 proteins and 5 small nuclear RNAs. Assembly of the spliceosome requires identification of the 5’ splice site by the U1 snRNP and a branch point adenine by the U2 snRNP. After the spliceosome is assembled, the hydroxyl group of the branch point adenine is used to perform a nucleophilic attack and covalently link to a guanosine at the 5’ splice site forming an intronic lariat. The exposed 3’ hydroxyl group of the upstream exon is then able to be covalently linked to a guanosine in the 3’ splice site of the downstream exon, joining the two exons. After splicing is completed a complex of eukaryotic initiation factor 4A3, MAGOH, Y14 and metastatic lymph node 51 are deposited 24 nucleotides upstream of the exon-exon junction. This Exon Junction Complex (EJC) aids in the export of mature mRNA from the nucleus to the cytoplasm and plays a role in degradation of mRNA transcripts with premature stop codons. The splicing of pre-mRNA transcripts can be altered by cells to produce different isoforms of a gene. However, mutations can also disrupt normal splicing of a gene to produce a deleterious isoform, such as is seen in Duchenne Muscular Dystrophy.
3’ Polyadenylation

The final modification needed for production of eukaryotic mRNA is the addition of a polyadenylate (poly(A)) tail. The addition of the poly(A) tail is intrinsically linked to the termination of transcription by RNA polymerase II, which facilitates assembly of the 3’ processing complex via its C-terminal domain. The cleavage and polyadenylation specificity factor (CPSF) protein complex recognizes the AAUAAA polyadenylation sequence of the pre-mRNA transcript and recruits cleavage stimulation factor, cleavage factor 1 and 2, poly(A) polymerase (PAP), and poly(A) binding protein (PAB2). The activity of the cleavage factors is inhibited in the absence of PAP, ensuring tight coupling of pre-mRNA cleavage and polyadenylation. The site of cleavage is not the same on each copy of a transcript. However, there is a strong preference for cleavage after a CA dinucleotide 10 to 30 nucleotides downstream of the polyadenylation signal. Mutation of the dinucleotide can significantly alter the efficiency of 3’ processing, as is seen in the case of a mutation to a CA dinucleotide in the prothrombin gene that causes hereditary thrombophilia due to more efficient production of prothrombin mRNA. The length of the poly(A) tail is determined by the duration of PAP association with CPSF and limits the number of added adenines to approximately 250, which is shortened in the absence of PAB2. The binding of PAB2 along the poly(A) tail promotes nuclear export and recruitment of translation initiation factors in the cytoplasm.

Post-transcriptional Regulation of mRNA

Once the mRNA has been transcribed the cell can still exert control over translation of the encoded protein in a variety of ways. To prevent wasteful energy spent
translating unwanted mRNA, cells exhibit a strong preference for regulating the initiation of translation over other mechanisms, such as transcript degradation. Most of these pathways involve the two untranslated regions (UTR) located at the 5’ and 3’ of the coding sequence.

Role of the Untranslated Regions

The importance of UTRs is highlighted by the high degree of localized evolutionary conserved regulatory elements \(^{13,14}\). Furthermore, a large expansion in the UTRs of genes is observed in higher order organisms, indicating that morphological complexity is perhaps driven by regulatory patterning rather than an increase in protein diversity \(^{15,16}\).

5’ Untranslated Region

As mentioned above, under certain cellular stresses, such as hypoxia, there is a switch away from cap-dependent translation. A subset of cellular mRNAs possess internal ribosome entry sites (IRES), which allow for ribosome formation and translation of the encoded protein under stress conditions \(^{17}\). This strategy is also responsible for transcription of cell cycle proteins like PITSLRE (p58), which is involved in mitotic spindle formation at the G2/M checkpoint \(^{18}\). In addition, IRES-mediated translation is a key driver of protein expression in apoptotic cells as the cap-dependent translation factors are cleaved by apoptotic caspases, allowing cells to continue producing the proteins necessary for the apoptotic process \(^{19}\).
Another mechanism for driving cap-independent translation has been observed for a small subset of transcripts which were shown to bind eukaryotic initiation factor 3 (eIF3) to stem-loop structures in the 5’ UTR. Unlike IRES, these eIF3-binding sites exhibited the ability to promote or inhibit translation of transcripts of c-Jun and B cell translocation gene 1, respectively. Further studies showed that the binding of eIF3 to the 5’ UTR is mediated by the reversible nucleotide modification N^6-methyladenosine (m6A) and that a single m6A in the 5’ UTR was sufficient for eIF3 to bypass cap-dependent translation.

The secondary structure of the 5’ UTR can also affect the efficiency of translation. The 5’ UTR tends to be less structured than the open reading frame, and transcripts with highly structured 5’ UTRs produced less protein than transcripts with unstructured 5’ UTRs. The helicase activity of eIF4A is required to unwind these highly structured regions. However, the binding of recognition proteins to the secondary structures in the 5’ UTR, such as iron response elements of ferritin and ferriportin, can prevent cap-dependent translation machinery assembly and subsequent unwinding by eIF4A. These recognition proteins are often responsive to environmental stimuli, such as the availability of cytoplasmic iron, and dynamically control the translation of their target transcripts.

3’ Untranslated Region and Poly(A) Tail

Nearly all eukaryotic mRNA is translated in pseudo-circular conformation due to the interaction of the eIF4G subunit of the cap-binding complex with PAB2 on the
poly(A) tail. Decreasing the distance between the cap-binding complex and the stop codon may improve translation by increasing the rate of ribosome recycling 25.

While the function of 5’ UTR is primarily to regulate translation initiation, the 3’ UTR of eukaryotic mRNA has a more diverse set of functions. The 3’ UTR, like the 5’ UTR, is capable of regulating the initiation of translation. Indeed, some of the same motifs discussed above, such as the iron response elements, can also exert their effects on translation when present in the 3’ UTR 26. In addition, the hairpin structures responsible for the alternative decoding of the “UGA” stop codon as selenocysteine are present in the 3’ UTR of selenoproteins 27. However, the primary function of the 3’ UTR is to provide binding sites for a variety of proteins that regulate mRNA turnover and sequence-specific transcript silencing.

mRNA Stability and Turnover

The level a given protein in a cell is often correlated with the number of mRNA transcripts that encode that protein 28. Transcript expression levels are determined by the balance of transcription and degradation. Like transcription, mRNA turnover must be a robust and tightly regulated process for cells to maintain mRNA expression levels at a steady state and to alter production of encoded proteins as needed.

mRNA Degradation

mRNA transcripts are constantly attacked by cytoplasmic nucleases. The 5’ end of mRNA transcripts are largely protected from exonuclease-mediated degradation by the presence of the 5’ cap. However, there are multiple deadenylases, such as poly(A)
ribonuclease, that specifically target the poly(A) tail by recognition of the 5’ cap, PAB2 or other RNA binding proteins. The removal of the poly(A) tail often serves as a trigger for the rapid inactivation of the mRNA transcript by removal of the 5’ cap and associated translational machinery via decapping protein 2 (DCP2) \(^{29}\). The decapped mRNA is then degraded from both the 5’ and 3’ ends by cytoplasmic exonucleases \(^{28}\).

Degradation of mRNA may also be initiated by endonuclease attacks. Only five such endonucleases have been identified, and the specificity of their activity is still under investigation. However, the activation of endonuclease-mediated mRNA degradation seems to be triggered in response to stimuli such as cellular or endoplasmic reticulum stress \(^{30}\). The products of endonucleolytic cleavage of mRNA are further degraded by the same 5’ and 3’ exonucleases discussed above.

**Determinants of mRNA Stability**

The stability of mRNA is influenced by factors intrinsic to the transcript, such as destabilizing AU-rich elements (ARE) and GU-rich elements (GRE), as well as extrinsic regulatory agents, such as RNA binding proteins. In addition, the effects of these factors may be linked to the translation, or lack thereof, of the mRNA transcript.

ARE and GRE are “AUUUA” and “UGUUUGUUGU” sequences, respectively, that occur in uridine enriched stretches of 3’ UTRs. These sequences are recognized by ARE- or GRE-binding proteins, which typically induce rapid degradation of the bound transcript via decapping \(^{31,32}\). However, one ARE-binding protein family, ELAV-like which is also known as human antigen, increases mRNA stability after binding to the ARE. The dysregulation of ARE-binding proteins has clinical implications in cancer as
there is significant enrichment of ARE in genes involved in cell cycle and proliferation, many of which are classified as protooncogenes \(^\text{33}\).

The translation status of an mRNA transcript has a profound impact on its stability. The principal determinant of mRNA stability was recently shown to be the stochastic competition between the 5’ cap-binding translation initiation factors and decapping enzymes \(^\text{34}\). In addition, the efficiency of translation also affects the half-life of mRNA transcripts. The composition of codons in the mRNA transcript and the relative abundance of the cognate transfer RNA (tRNA) in the cell can limit the rate of translation. The inclusion of rare codons in an mRNA transcript decreases protein production and causes ribosomal stalling \(^\text{35}\). This stalling can be sensed by RNA binding proteins such as DEAD-box helicase 6 (DDX6) which promotes removal of the 5’ cap and subsequent mRNA transcript degradation \(^\text{36}\).

**Silencing by microRNA**

In addition to the post-transcriptional regulators discussed above, most eukaryotes possess an RNA-directed transcript silencing system that identifies its targets by Watson-Crick base pairing between a ~22 nucleotide single stranded RNA, called a microRNA (miRNA), and a complementary target site on an mRNA. The activity of miRNA provides an additional layer of dynamic control over mRNA translation and stability. However, the primary physiological role of miRNA is to fine tune gene expression. Many miRNA are expressed in specific types of cells, such as the endothelial cell specific miR-126, while others are almost ubiquitously expressed like miR-3960 \(^\text{37–39}\).
microRNA Biogenesis

RNA polymerase II transcription of genes with short hairpin structures located in intronic regions or independent non-coding genes, called primary microRNA (pri-miRNA), results in cleavage by nuclear Microprocessor complex. The Microprocessor complex is made up the catalytic Drosha subunit and two structural DGCR8 subunits. All three subunits function in the recognition of the stem-loop structure of the pri-miRNA. Drosha recognizes the single stranded RNA (ssRNA)- double stranded RNA (dsRNA) junction. The size of Drosha and the helical structure of the dsRNA ensures that pri-miRNA are cleaved 11 nt from the ssRNA-dsRNA junction and 22 nt from the apical loop with a 2 nt overhang on the 3’ end to form the precursor-miRNA (pre-miRNA).

The pre-miRNA is then exported from the nucleus after binding to Exportin 5 and RAs-related Nuclear protein (RAN)-GTP. In the cytoplasm, pre-miRNA is further processed by Dicer, which recognizes the 5’ end of the pre-mRNA. Again the helical structure of the dsRNA allows Dicer to specifically cleave the both strands of the pre-miRNA 22 nt from each end to produce two mature miRNA.

The RNA-induced Silencing Complex

The mature miRNA remains double stranded until they are loaded into the RNA-induced Silencing Complex (RISC). The mechanisms and proteins involved in the loading of miRNA duplexes into Argonaute (Ago) proteins, the effector subunit of the RISC, is not well conserved between mammals and lower order organisms like Drosophila. In Drosophila, Dicer-2 (dcr-2) and the dsRNA binding protein R2D2 coordinate the loading of the miRNA into Ago. However in humans and mice, Dicer is
not needed for loading of the miRNA duplexes, although a Dicer-Ago2-TRBP (TAR-RNA binding protein) complex has been described\textsuperscript{45,46}. The bulkiness of the miRNA duplex necessitates a energetically unfavorable conformational change in Ago that is driven by adenosine triphosphate (ATP) hydrolysis by Heat shock protein 90 (Hsp90) in all organisms\textsuperscript{47}.

Once the miRNA duplex is loaded in to Ago, one of the strands must be ejected from the complex to allow for scanning of target mRNAs. Which strand of the miRNA duplex remains bound in Ago depends primarily on the stability of the initial interaction of the 5’ monophosphate nucleotide with the middle (MID) domain of Ago, which shows a preference for binding adenine or uridine over guanosine or cytosine\textsuperscript{48}. Removal of the unincorporated passenger strand is driven by pressure from the Ago Piwi-Argonaute-Zwille (PAZ) domain that is displaced by loading of the miRNA duplex. In human Ago2 is the only Ago protein that has the ability to endonucleolyticaly cleave target RNAs, and cleavage of the passenger strand facilitates its release from the RISC\textsuperscript{49}.

\textit{Mechanisms of microRNA Activity}

Once the guide strand of miRNA has been loaded, the RISC begins scanning cytoplasmic transcripts including mRNA for target sites that are complementary to the loaded miRNA. Recognition is mediated by nucleotides 2 – 8 of the guide miRNA, called the seed sequence\textsuperscript{50}. This seed sequence may be conserved in multiple miRNA, forming a miRNA family. Over half of the human transcriptome is targeted by miRNA,
and each miRNA or miRNA family can targets hundreds of different mRNA transcripts.

In their canonical role, miRNA repress the expression of targeted mRNA. This repression can be accomplished by two different mechanisms. When the RISC binds to a miRNA target site that is complementary to only the seed sequence, which is the predominant type of target site in animals, the primary means for miRNA-mediated silencing is the recruitment of mRNA degradation machinery. Ago proteins that are loaded with a miRNA can form stable complexes with the trinucleotide repeat containing protein 6 (TRN6). After binding to the cognate miRNA target site, the Ago-TRN6 complex interacts with poly(A) binding proteins to recruit deadenylases and the carbon catabolite repressor 4-Negative on TATA complex (CCR4-NOT). CCR4-NOT in turn recruits the DDX6 decapping enzyme. In addition, DDX6 competes with translation initiators to reduce protein expression prior to decapping.

In contrast to the translational repression mediated by the RISC binding to the seed complementary sequence, binding to target sites with extensive or perfect complementarity to the entire miRNA induces cleavage by Ago proteins. In humans, Ago2 is the only Ago protein that has RNA cleaving activity, and thus far only 20 endogenous mRNA transcripts have been experimentally proven to be cleaved by Ago in all mammals. However, the cleavage of mRNA by the RISC is the predominant mechanism of action in plants. Importantly, this mechanism has been extensively exploited in the design of short interfering RNA (siRNA). Extensive or perfect complementarity positions the substrate mRNA within the catalytic PIWI domain of Ago2, which cleaves the phosphodiester backbone between the nucleotides.
complementary to nucleotides 10-11 of the loaded miRNA. Once the mRNA is cleaved and degraded the miRNA-loaded RISC is freed to search for additional targets.

**Translation of mRNA to Protein**

mRNA serves as the intermediate step between the storage of gene in the DNA and the effector of the gene, protein. Like transcription, translation is a tightly controlled process, and the translation of an mRNA sequence to a peptide sequence is the final regulatory checkpoint at which a cell can regulate mRNA. Translation in eukaryotes is highly coordinated and requires specific factors for translation initiation, peptide elongation, and termination. Translation also serves as a key quality control checkpoint for detecting and disposing of aberrant mRNAs.

**Initiation of Translation**

As discussed above, the initiation of translation plays a major role in the stability of an mRNA transcript. The synthesis of proteins requires a large energy investment by a cell. Halting the initiation of translation is the most effective way of preventing this loss of energy during times of cellular stress or nutrient deprivation. It is not surprising therefore that cells tightly regulate the drivers of translation initiation.

**Eukaryotic Translation Initiation Factors**

The primary method of translation initiation in eukaryotes is dependent on the 5’ cap of the substrate mRNA. Cap-dependent translation initiation requires the independent preassembly of eukaryotic initiation factors (eIF) on the substrate mRNA,
the 40S ribosomal subunit, and methionine transfer RNA (tRNA\textsuperscript{Met}). The 5’ cap is recognized by the eIF4F complex, composed of the IF4A, eIF4G, and eIF4E subunits. Once bound, eIF4F hydrolyzes ATP to unwind a small stretch of the 5’ UTR. This “activated” mRNA is prepared for loading into the pre-initiation complex and subsequent translation.

The pre-initiation complex is formed by the binding of eIF3 and eIF1A to the 40S ribosomal subunit. The complex is then charged by the binding of the guanosine triphosphate (GTP)-eIF2-tRNA\textsuperscript{Met} ternary complex at the ribosomal peptidyl site (P-site)\textsuperscript{55}. The charged pre-initiation complex, also known as the 43S ribosomal subunit, then associates with the activated mRNA via interactions between eIF3 and eIF4G.

Translation initiation is regulated by inactivation eIF4E or eIF2. eIF4E is the target of three eIF4E-binding proteins (4E-BP) that are regulated by environmental signals, such as growth factors, as well as stressors like nutrient deprivation. The reversible binding of 4E-BP prevents eIF4E binding to eIF4G to form the eIF4F complex. Phosphorylation of 4E-BP in response to growth factors releases the bound eIF4E and allows for translation initiation\textsuperscript{56}. This phosphorylation is carried out by the FKBP12-rapamycin associated protein/mammalian target of rapamycin (FRAP/mTOR), which is a primary sensor of nutrient availability and integrates initiation of translation with a variety of other cellular processes\textsuperscript{57}.

Unlike the singular kinase regulating eIF4E, there are four known regulatory kinases that target mammalian eIF2. Three of these sensors, general control nonderepressible 2 (GCN2), hemin-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK), detect states of cellular stress or nutrient deprivation \textsuperscript{58}. The fourth
sensor, double-stranded RNA-activated protein kinase (PKR), is primarily activated in response to viral infection but can also be activated by synthetic mRNA. Phosphorylation of eIF2 sequesters eIF2B, which prevents recycling of GDP-eIF2 to the active GTP-eIF2 and subsequent formation of the ternary complex.

Ribosome Assembly

After binding to the mRNA, the pre-initiation complex begins to move along the mRNA in the 3’ direction. This movement is aided by the ATP-dependent helicase activity of eIF4A. The complex continues to scan along the mRNA until it encounters an “AUG” start codon that is recognized by the tRNA\textsuperscript{Met} anti-codon loop in the P-site of the small ribosomal subunit. Recognition of the start codon triggers removal of the bound initiation factors and ribosome assembly.

The joining of the large ribosomal subunit to the mRNA-bound small ribosomal subunit requires another translation initiation factor, eIF5. The presence of eIF5 promotes the self-hydrolysis of GTP-eIF2 to GDP-eIF2, and the liberated phosphate ion induces a conformational change in eIF5 that arrests the smaller ribosomal subunit on the start codon. The recognition of the start codon also induces a conformational change that releases eIF1 from the small ribosomal subunit, allowing for the large ribosomal subunit to associate with the small ribosomal subunit. Formation of the complete ribosome prompts hydrolysis of GTP on eIF5 that reduces its affinity for the ribosome.
Peptide Elongation

The assembled ribosome is now poised to translate the rest of the mRNA. The continued translation decodes the mRNA into a peptide sequence and is coordinated by a family of eukaryotic elongation factors (eEF). The rate of translation also plays a role in the efficiency of protein production, balancing speed of synthesis with the time required to properly fold the protein products. All of these processes are monitored by the cell to prevent translation of degraded or degenerate mRNAs through No-Go Decay.

Decoding of mRNA

Translation proceeds one trinucleotide codon at a time, and each codon is recognized by a single species of tRNA that possesses the complimentary anti-codon. The amino acid loaded-tRNAs (aa-tRNA) are directed to the aminoacyl site (A-site) of the ribosome as a complex with eEF1A and GTP. Loading of the complementary aa-tRNA induces a conformational change in eEF1A that induces hydrolysis of the GTP to GDP and subsequent dissociation from the aa-tRNA. A peptide bond is rapidly formed between the last amino acid in the peptide chain and the amino acid on the aa-tRNA, forming a peptidyl-tRNA. The now uncharged tRNA in the P-site and peptidyl-tRNA in the A-site induce a GTP-dependent translocation of the mRNA within the ribosome to reposition the tRNAs in the exit site (E-site) and P-site, respectively. The hydrolysis of GTP that drives translocation is carried out by eEF2, which is dissociated from the ribosome by the resulting conformational change.\textsuperscript{62}
Effects of Translation Rate

The rate at which the translation of mRNA occurs is largely determined by the codon composition of the transcript. Optimal codons that are recognized by relatively abundant tRNAs are translated at a higher rate than rare codons with less abundant tRNAs. The inclusion of rare codons can induce a stall in translation and reduce the amount of protein produced from an mRNA \(^{35}\). However, both optimal and rare codons are found to be evolutionarily conserved, indicating a need for ribosomal pausing. Indeed, structured regions of proteins, such as \(\alpha\)-helices, often exhibit a pattern of optimal and rare codons that aids in the co-translational folding, while less structured regions tend to be enriched in optimal codons \(^{63}\).

No-Go Decay

While some ribosomal pausing is important to proper protein folding, excessive pausing may be indicative of degraded or degenerate mRNA. Removing these mRNAs and liberating the bound ribosomes is achieved by a mRNA surveillance pathway called No-Go Decay. Details of the mechanism that induces this system are not currently well understood, however, Dom34 and Hbs1 proteins have been shown to be involved in No-Go Decay in yeast \(^{64}\). Degradation of the mRNA transcript is driven by cleavage within the mRNA exit tunnel of the ribosome and requires at least three stalled ribosomes \(^{65}\).
Translation Termination

Proper translation of the mRNA requires that decoding terminates at the final codon. This process, like initiation and elongation is regulated by a small set of proteins called eukaryotic releases factors (eRFs). The activity of the eRFs is tied to recognition of the final codon in the coding region of the mRNA, triggers the release of nascent peptide to prevent production of truncated proteins, and disassembles the ribosome. However, cells must also be able to detect when errors in translation termination occur and destroy the defective mRNA.

Stop Codons

Like the initiation of translation, the site of translation termination is also encoded within the mRNA as Stop codons. Unlike the Start codon, there are three codons that signal for translation to stop: Amber (UAG), Ochre (UAA), and Opal (UGA). In humans and other higher order eukaryotes, UGA is the most commonly used stop codon \(^{66}\). However, in highly expressed genes UAA is more frequently used, possibly due to the higher efficiency of termination than UAG or UGA \(^{67,68}\).

In a small set of proteins, UGA encodes for a selenocysteine rather than Stop. This alternative decoding is mediated by a nearby selenocysteine incorporation sequence \(^{69}\). Expression of selenoproteins can be regulated by the abundance of selenocysteine-tRNA; the lack of environmental selenium causes the UGA to be decoded as Stop and prematurely terminates translation \(^{70}\).
Eukaryotic Release Factors

Recognition of all three stop codons in eukaryotes is mediated by eRF1, which contains a codon recognition region similar in structure to the anticodon loop of tRNA. Before entry into the ribosome, eRF1 forms a complex with eRF3 and GTP. Like tRNA, eRF1 binds to the stop codon in the A-site of the ribosome, which induces hydrolysis of the complexed GTP via eRF3 and positions the Gly-Gly-Gln motif of eRF1 to catalyze the release of the nascent peptide from the peptidyl-tRNA in the P-site of the ribosome. The eRF1/eRF3 complex can then promote disassembly of the large ribosomal subunit, a process that is greatly accelerated by association with ATP Binding Cassette Subfamily E Member 1 (ABCE1). The small ribosomal subunit remains bound to the mRNA until the deacetylated tRNA is removed by eIF1, which also initiates the reformation of the preinitiation complex.

Nonsense-Mediated Decay

Mutations that create a stop codon from a sense codon are called nonsense mutations. The inclusion of a premature stop codon in an mRNA can cause the production of a truncated and potentially deleterious protein product. Cells possess an mRNA quality control system that targets transcripts that are not completely translated for degradation. In mammals this nonsense-mediated decay pathway relies on the exon junction complexes (EJC) deposited on the mRNA during splicing. The EJC is normally displaced during translation by ribosomes, but premature termination codons at least 50 nt upstream of the last exon junction will allow the EJC to persist on the mRNA transcript. The remaining EJC is recognized by complex of suppressor with
morphological effect on genitalia 1 (SMG1), Regulator of nonsense mediated decay 1 (UPF1), eRF1, and eRF3 (SURF). Binding of SURF to the EJC recruits UPF2 and UPF3b and triggers dissociation eRF1 and eRF3 along with activation of the helicase activity of UPF1. UPF1 proceeds along the transcript removing mRNA-binding proteins to enable endonucleolytic attack by SMG6. The cleaved mRNA is then rapidly degraded by mRNA decay due to decapping enzymes and deadenylases that are recruited by the activated UPF1, UPF2, and UPF3b.

Nonstop-Mediated Decay

In addition to the premature translation termination of mRNAs with nonsense mutations, cells must be able to recognize transcripts that fail to terminate translation due to destruction of the stop codon by a nonstop mutation or premature polyadenylation. Nonstop mutations can lead to accumulation of ribosomes on the defective mRNA as eRF1 is not able to induce ribosomal release. In addition, the stalled ribosomes increase the transcript’s resistance to 3’ to 5’ exonuclease degradation. In mammals the stalled ribosomes are recognized by a complex of HBS1-like translation GTPase (HBS1L; Hbs1) and pelota mRNA surveillance and ribosome rescue factor (PELO; Dom34), which is also involved in No-Go decay discussed above. The Hbs1-Dom34 complex interacts with the vacant A-site of the ribosome in a codon-independent manner. Once bound to the ribosome, the Hbs1-Dom34 complex recruits mRNA degradation machinery. Unlike normal mRNA degradation, Nonstop decay does not induce deadenylation of the transcript and instead promotes attack by endonucleases.
Chapter 2

Design and Synthesis of Synthetic mRNA for Therapeutic Applications

Nucleic Acid Therapies

Replacing a defective, pathogenic gene using exogenous nucleic acids has the potential to provide a curative treatment for many human diseases. The promise of these gene therapies has driven research on the design and delivery of these treatments for over 50 years. However, the applications of nucleic acid therapies extend beyond correction of disease-causing mutations by gene therapy.

Overview of Nucleic Acid Therapies

The ability to deliver a specific gene to a cell enables manipulation of the cell’s activity, metabolism, and even phenotype. Much of the research on nucleic acid therapies has focused on the delivery of DNA to the target cells in the form of plasmids or viral vectors. This work has produced multiple successes, including a treatment for hereditary blindness 79 and the development of chimeric antigen receptor T-cell (CAR-T) therapy for lymphomas and leukemia 80. However, the delivery of DNA to cells is not without limitations and dangers.

Because the delivered DNA must be transcribed to produce the therapeutic effect, it must bypass both the cellular and nuclear membranes. The breakdown of the nuclear membrane during mitosis allows cytoplasmic DNA to be incorporated within the
nuclei of the daughter cells. However, this largely precludes the use of DNA-based therapeutics in non-dividing cells except when delivered using a lentiviral vector.\textsuperscript{81}

The use of retroviral vectors also allows for permanent integration of the transgene into the genome of a cell. However, many retroviruses integrate randomly into the host DNA and have to potential to disrupt normal gene expression. The danger of insertional mutagenesis was highlighted after two patients treated with a retroviral therapy for X-linked severe combined immunodeficiency developed leukemia.\textsuperscript{82,83} The shift toward Adeno-associated virus (AAV) vectors in recent years has been driven in part by the selective integration of AAV transgenes at a specific locus on chromosome 19.\textsuperscript{84}

**Advantages of mRNA Therapeutics**

Synthetic mRNA has recently begun to gain favor over DNA or viral vectors for transient applications and therapies. The rise of mRNA therapeutics can be attributed to the intrinsic advantages they hold over DNA vectors therapies. Unlike DNA vectors, synthetic mRNAs can produce the encoded protein in any cell type, even non-dividing cells, because synthetic mRNAs are immediately translated in the cytoplasm and do not require translocation to the nucleus. Synthetic mRNA vectors carry no risk of insertional mutagenesis or recombination. Furthermore, the production of synthetic mRNA is carried out in a cell-free system that minimizes the potential for contamination, is much simpler, cheaper and faster than the production of viral vectors, and can be quality controlled to ensure uniformity and purity by high performance liquid chromatograph (HPLC).\textsuperscript{85}
**Innate Immune Response to Exogenous RNA**

The viability of synthetic mRNA as a therapeutic has long been hindered by the induction of the innate immune response. Barrier cells, such as vascular endothelial cells, and immune sentinel cells, like dendritic cells, express membrane bound Toll-like receptors (TLRs) that act as pattern recognition receptors (PRRs) for a variety of ligands, including extracellular and endocytosed RNA. In addition, nearly every type of mammalian cell possesses cytosolic PRRs capable of detecting viral RNA or synthetic RNA. Activation of these various PRRs enable cells to initiate a variety of antiviral responses. 

**Toll-like Receptors**

The first PRRs that encounter exogenous RNA are the endosomal TLR3, TLR7, and TLR8. The activation of these TLRs leads to the establishment of an antiviral cellular state, by inducing the expression of various cytokines that produce a localized inflammatory response and stall translation. TLR7 and TLR8 are both capable of sensing ssRNA that is guanosine- or uridine-enriched. TLR7/8 is bound by a cytosolic adapter protein, myeloid differentiation primary response 88 (MYD88), and stimulation of TLR7/8 leads to activation of IL-1 receptor-associated kinase (IRAK) family. IRAK phosphorylates TNF receptor associated factor 6 (TRAF6), which in turn activates the IκB kinase (IKK) complex via transforming growth factor beta-activated kinase 1 (TAK1). IKK subsequently activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by ubiquitination of inhibitor κB (IκB). NF-κB then translocates to the nucleus and activates the transcription of pro-inflammatory cytokines.
such as tumor necrosis factor alpha (TNFα), interleukin 12 (IL-12), and interleukin 6 (IL-6). In addition to activation of the TNFα pathway, MYD88 allows IRAK1 to phosphorylate Interferon Regulatory Factor 7 (IRF7). IRF7 then induces expression of interferon alpha (IFNα), which promotes expression of genes involved in anti-viral defense in an autocrine and paracrine manner²⁰.

In contrast to TLR7/8, TLR3 recognizes dsRNA. TLR3 utilizes a different cytoplasmic adapter, TIR-domain-containing adapter-inducing interferon-β (IFNβ) (TRIF)⁰. Stimulation of TLR3 allows TRIF to bind and activate TRAF6, resulting in the nuclear translocation of NF-κB. However, TRIF also mediates the phosphorylation of IRF3 via TANK binding kinase 1 (TBK1)⁸⁹. Phosphorylated IRF3 induces expression of IFNβ, which induces the same antiviral response as INFα.

_Cytosolic dsRNA Sensors_

Unlike the TLRs that are found in barrier cells and immune sentinel cells, the expression of cytosolic sensors is nearly ubiquitous. The cytosolic sensors induce 3 distinct responses upon stimulation: induction of an antiviral state, translational inhibition, and ribonuclease (RNase)-mediated apoptosis (Figure 2.1). To control the latter two responses, their activation is potentiated by interferon signaling.
Figure 2.1. The components of the RNA-sensing innate immune response

Diagram of the various pattern-recognition receptors and the downstream signaling cascade (blue) involved in the detection of RNA. Stimulation of the innate immune response by RNA produces three primary effects (dark grey): inhibition of protein translation, induction of an antiviral state, and RNase-mediated apoptosis.
Production of interferons is mediated by two homologous cytosolic sensors of dsRNA, retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), which belong to the RIG-I-like receptor family. RIG-I is most strongly stimulated by short (<300 bp) dsRNA with a 5’ triphosphate, a feature common in viral genomes that lack the 5’ cap. The ligands for MDA5, however, are not well established, though it is known to be stimulated by long stretches (>2 kb) of poly(I:C) dsRNA and mRNA lacking ribose 2’-O-methylation. Recent work has also demonstrated that MDA5 is stimulated by mitochondrial dsRNA, which might escape into the cytoplasm during viral infections. Signal transduction from both RIG-I and MDA5 is carried out by an adaptor protein, mitochondrial antiviral signaling (MAVS). MAVS functions much like the TLR3 adapter TRIF; activation of MAVS induces the expression of INFα via TRAF6 and NF-κB translocation via the IKK complex.

PKR is another cytosolic dsRNA sensor. PKR is robustly expressed in all cell types, but its expression is strongly induced by type 1 interferon (IFNα/IFNβ). Upon dsRNA binding PKR dimerizes, autophosphorylates, and inhibits translation initiation by phosphorylating eIF2α and prevents charging of the small ribosomal subunit with tRNA\textsuperscript{Met}.

The final cytosolic sensor of dsRNA is the oligoadenylate synthetase family (OAS), which is composed of three catalytically active members (OAS1, OAS2, and OAS3) in humans. Activation of OAS1 can be triggered by short dsRNA structures as short as 17 bp in length, while OAS2 requires dsRNA at least 35 bp in length for robust activation. The size specificity of the ligand dsRNA is likely determined by the presence of 1, 2, or 3 copies of the OAS homology domain in OAS1, OAS2, or OAS3,
respectively. dsRNA binding induces a conformational change that exposes the catalytic residues of OAS and allows for the production of 2',5'-oligoadenylylate second messengers. The length of the 2',5'-oligoadenylates varies among OAS1, OAS2, and OAS3, but all three are capable of producing the trimers needed to activate the 2'-5' oligoadenylate synthetase-dependent ribonuclease RNase (RNAse L), which endonucleolytically cleaves ssRNA in uridine-rich regions to produce 5' hydroxyl and 3' phosphate products that can be recognized by RIG-I and further induce or prolong interferon expression. RNase L also specifically cleaves the 18S ribosomal RNA and induces caspase-dependent apoptosis due to release of mitochondrial cytochrome C.

Incorporation of Chemically Modified Nucleotides

Given the preponderance of secondary structure present in mRNA, cells must possess a means of preventing activation of the innate immune response by their own mRNA. The processing of pre-mRNA to mRNA places several identifying marks upon cellular mRNA, such as the 5' cap, and provides an opportunity for mRNA binding proteins to attach before export to the cytoplasm. While synthetic mRNA is commonly capped, robust activation of the innate immune response is still seen. Recently, incorporation of various chemically-modified nucleotides (Figure 2.2) during in vitro transcription has been shown to improve protein production and reduce immunogenicity of synthetic mRNA. These modified nucleotides are also found in cellular mRNA, but their abundance compared to unmodified nucleotides is extremely low, typically less than 1%.
Figure 2.2. Chemical structure of modified nucleotides commonly incorporated in synthetic mRNA.

Representative unmodified and chemically-modified nucleotides produced by post-transcriptional modification of eukaryotic mRNA. Inclusion of these modified nucleotides during *in vitro* transcription allows for direct incorporation into synthetic mRNA in stoichiometrically controlled ratios.
Avoidance of Innate Immune Response

The endosomal TLRs are the first innate immune sensors encountered by synthetic mRNA. Initial screening showed that a variety of nucleotide modifications, including 6-methyladenosine (m6A), 5-methylcytosine (m5C), 2-thiouridine (s2U), and pseudouridine (Ψ), could prevent recognition by TLRs\textsuperscript{102}. Recent work has also shown that N\textsuperscript{1}-methylpseudouridine (m1Ψ) modification prevents activation of TLR3\textsuperscript{103}. Moreover, incorporation of Ψ, m5C, or m1Ψ also increased the expression of proteins encoded by the synthetic mRNA\textsuperscript{104}. This increase in translation is due, at least in part, to the decreased recognition of the modified mRNA by PKR\textsuperscript{105,106}. Interestingly, s2U also prevented phosphorylation of eIF2α by PKR, however the protein production was decreased compared to unmodified mRNA. m6A modification did not increase the production of protein and also failed to prevent PKR activation\textsuperscript{104,105}.

In cells lacking TLRs, RIG-I is the primary innate immune sensor for inducing interferon expression in response to exogenous mRNA as the Cap1 structure prevents activation of MDA5\textsuperscript{92}. Although Ψ-modified mRNA is recognized by RIG-I, it prevents RIG-I activation by inhibiting the conformational change necessary for RIG-I to bind to MAVS and induce expression of INFβ\textsuperscript{104,107}.

Unmodified synthetic mRNA also induce severe cytotoxicity in some cell types, likely due to the production of second messenger 2',5'-oligoadenylates from OAS1 and activation of RNase L. However, incorporation of Ψ, m6A, or s2U modifications in mRNA prevented the activation of OAS1. Furthermore, Ψ-modified mRNA is more resistant to cleavage by activated RNase L \textit{in vitro}\textsuperscript{108}.
Changes in RNA Secondary Structure

Since the sensors of the innate immune system do not recognize specific nucleotide sequences, but rather mRNA secondary structure, the ability of nucleotide modifications to avoid detection must be mediated by changes in the mRNA structure. In fact, addition of methyl group in m6A has been shown to greatly increase the energy needed for m6A·U Watson-Crick base pairing compared to A·U and disrupts dsRNA structures\textsuperscript{109}. This destabilization is supported by the enrichment of m6A nucleotides in regions of dsRNA to ssRNA transition\textsuperscript{110}. Incorporation of Ψ promotes base stacking of nearby nucleotides into A-form RNA, a compacted right-handed double-helix. The compacting is due to stabilization by the N1 interacting with the ribose backbone\textsuperscript{111}. s2U modification also increase bases stacking by making the C3′-endo conformation of the ribose sugar more energetically favorable, thereby reducing the distance between adjacent bases\textsuperscript{112}. Like Ψ and s2U, m5C is also thought to increase base stacking, but the denser stacking is driven by increased hydrophobicity rather than direct stabilization of backbone conformation\textsuperscript{113}.

Alternative Decoding of Modified Codons

In addition to altering the secondary structure of the mRNA transcript, the inclusion of modified nucleotides can also alter the Watson-Crick base pairing energies. m6A is known to decrease the energetic favorability of all potential base pairings, and formation of m6A·U base pairs induces electrostatic tension due to steric clashing between the 6′-methyl group and N7 of the m6A\textsuperscript{110}. s2U increases the specificity of base pairing by stabilizing pairing with adenosine (s2U·A) and destabilizes pairing with
guanine (s2U-G). Formal studies examining the effect of the reduced wobble base pairing potential of s2U-G have not been performed, but s2U is commonly included in anti-sense RNA oligos and siRNA to reduce off-target binding. In contrast, Ψ exhibits significantly altered base pairing characteristics from unmodified uridine. Inclusion of Ψ in a Stop codon (ΨAA, ΨAG, or ΨGA) almost completely prevented transcription termination. Alternative decoding of this Nonstop codon varied depending on the codon sequence; ΨAA encoded serine or threonine equally, ΨAG primarily encoded serine, and ΨGA largely decoded to tyrosine. Whether Ψ induces alternative decoding of sense codons has not yet been determined, but the functionality of proteins produced by Ψ-modified synthetic mRNAs implies that any differences may be minimal. Currently, m5C produces no known alterations in base pairing stability or decoding of m5C-containing codons.

**Codon Optimization**

Synonymous codons encode for the same amino acid but differ in their sequence on the mRNA transcript. As such, they are recognized by different species of tRNA. The relative level of each tRNA in a cell varies by species and in response to metabolic activity and cellular stress. The importance of the relationship between codon frequency and tRNA abundance has produced several different measurements of codon usage bias.
Codon Usage Bias Indices

The first attempt at measuring codon optimality was based on the relative abundance of tRNA species. The use of cognate codons for the most abundant tRNA for each amino acid, the optimal codon, was found to correlate well with the protein levels of the gene in *Escherichia coli* \(^{118}\). The most common measurement of codon usage bias is the codon adaptation index (CAI), which is the geometric mean of the ratio of synonymous codons in given gene compared to usage of that codon in a set of highly expressed genes. CAI values range from 0 to 1, and genes with a CAI near 1 are predicted to be highly expressed \(^{119}\). A measurement of codon usage bias that relies on the expected codon frequency determined by the nucleotide composition of the measured gene has also been shown to capture the effect of codon optimality on protein abundance in *E. coli* without the need for a set of reference genes \(^{120}\). Therefore, engineering the sequence of synthetic mRNA to include a higher proportion of optimal codons may provide an additional opportunity for increasing the expression of the encoded protein.

Sequence Engineering for Protein Production

The degeneracy of the genetic code allows for an exceedingly large number of mRNA transcripts to produce identical proteins. Editing of synthetic mRNA or other gene vectors to contain a higher proportion of optimal codons can significantly increase the production of the encoded protein \(^{121,122}\). However, the generality of these results for expression in humans and other higher order metazoans requires further study as most
studies utilized bacterial or yeast models. In addition, codon usage bias in higher order eukaryotes is markedly smaller than in bacteria and yeast 123.

Codon optimization algorithms vary in the degree of codon replacement, ranging from complete replacement of all instances of an amino acid to adjustment of codon usage to the endogenous rate. Some programs also seek to conserve regions with a high proportion of rare codons as the reduced translation rate in these regions may be required for proper protein folding or modification 124. In addition, preserving rare codons in the beginning of the coding sequence has been shown to increase translation of proteins 125. Further studies, especially ones that utilize synthetic mRNA, are required to determine the true optimal codon usage.

Perhaps the clearest example of the functional effects of codon usage on expression is the switch in *E. coli* tRNA charging upon amino acid starvation. The preferential acylation of tRNAs that recognize rare codons enables efficient translation of mRNAs enriched with those codons, such as genes involved in amino acid synthesis 126. Importantly, a similar investigation using human cell lines found that genes involved in protein recycling are enriched with rare codons that enable preferential translation during amino acid starvation 127.

A similar change in the tRNA pool has also been found to drive preferential translation of proliferation-associated genes or differentiation-associated genes in normal and cancerous human cell lines. In particular, the abundance of tRNA$^\text{Met}$ was increased in proliferating cells 128. This phenomenon is supported by a study that demonstrated an increase in human epithelial cell proliferation after overexpression of tRNA$^\text{Met}$ 129. Perturbations of tRNA gene expression have also been shown to drive
metastasis in human cancer models\textsuperscript{130}. Therefore, it may be necessary to balance the codon optimality of a synthetic mRNA to ensure efficient translation without disrupting the availability of tRNA in a cell.

**Structural Components for Transcript Optimization**

As previously discussed, the maturation of eukaryotic pre-mRNA to mRNA involves extensive processing and modification. The translation machinery of eukaryotic cells built specifically to recognize the processed mRNA and target unprocessed or malformed mRNA for degradation. Therefore, incorporation of these elements, such as the 5’ cap and UTRs, into synthetic mRNA is required for an effective gene vector. The flexibility and \textit{in vitro} synthesis of synthetic mRNA provides a unique opportunity to explore different methods of including the desired structural components and study the effects of these components on the regulation of transcript stability and translation.

**5’ Cap**

The addition of the 5’ cap to synthetic mRNA is necessary for assembly of the canonical translation initiation complex. However, the simplified transcription machinery used for making synthetic mRNA does not enable co-transcriptional capping. The enzymatic addition of the 5’ cap can be carried out after completion of the \textit{in vitro} transcription reaction. In eukaryotes, 5’ capping is carried out by multiple enzymes that dephosphorylate the 5’ triphosphate, add a guanine nucleotide, and methylate the added guanine. However, virus have evolved capping enzymes that combine some or all of the requisite activity into a single protein\textsuperscript{131}. Many commercially available \textit{in vitro}
capping systems utilize a capping enzyme derived from Vaccinia virus that is capable of generating Cap0 but lacks methyltransferase activity. Cap0 is then converted into Cap1 by an included 2’-O-methyltransferase (Figure 2.3 A). The enzymatic capping method ensures that virtually all of the transcripts receive the 5’ Cap1.

The discovery of m7G cap analogs has allowed for co-transcriptional capping of synthetic mRNA. The use of analogs simplifies the mRNA synthesis process but does not guarantee the capping of every transcript. During IVT, regular guanine can be incorporated in place of the cap analog, producing 80% capped mRNA when used at a ratio of 4 m7GpppG to 1 guanine. The efficiency of the basic Cap0 analog m7GpppG (Figure 2.3 B) is further reduced by half due incorporation in the reverse orientation, with the m7G in the second nucleotide position. The design of cap analogs with 3’-O-methylation of the m7G was found to prevent incorporation in the reverse direction due to the inability of RNA polymerase to attach the next nucleotide to the 3’ hydroxyl (Figure 2.3 C). This Anti-Reverse Cap Analogs (ARCA) enables all of the capped transcripts to be translated, but capping efficiency is still driven by stoichiometric competition with guanine. Further modification of ARCA has produced new analogs capable of increasing translation initiation, transcript stability, and biotin-labeling of mRNA. However, all of the ARCA require subsequent methylation to produce Cap1 structures. The recent development of Cap1 and Cap2 analogs may enable to production of synthetic mRNA in a single reaction, but these analogs require a specific AG or GG dinucleotide initiator sequences for efficient incorporation.
Untranslated Regions

As previously discussed, the 5' and 3' UTR of mRNA transcripts play a key role in the regulation and stability of the mRNA. Because the coding region of synthetic mRNA is all that is necessary for the production of the desired protein, the flanking UTRs can be freely altered. Replacing the native UTRs of the synthetic mRNA with the UTRs of transcripts with exceptionally long half-lives, such as housekeeping genes, can confer the same effect to the synthetic mRNA. Conversely, the inclusion of UTRs with destabilizing elements can be used to reduce the half-life of the synthetic mRNA. In addition, the inclusion of some UTRs has been found to increase the translation efficiency without affecting the half-life of the synthetic mRNA.

Polyadenylation

In addition to 5' capping, eukaryotic mRNA also receives a poly(A) tail during pre-mRNA processing. The addition of the poly(A) tail to the synthetic mRNA is equally important for efficient translation. The simplest means of polyadenylating a synthetic mRNA is by including a stretch of adenines in the transcription template. Encoding the poly(A) tail within template allows for direct control over the length of the tail, although slippage of the RNA polymerase can produce slightly longer tails than encoded in the template. In addition, direct encoding allows for production of the tail during transcription. Alternatively, the poly(A) tail can be added post-transcriptionally by E. coli poly(A)-polymerase using commercially available kits. This enzymatic addition generally provides tails of 100 to 200 nucleotides in length.
Figure 2.3. Structure of mRNA 5' cap and cap analogs.

(A) The mRNA 5' cap consists of 5'-5' joining of m7G and the first transcribed nucleotide, forming Cap0. Methylation of the 2' hydroxyl of the first and second nucleotides occurs to procure the Cap1 and Cap2, respectively. (B) Structure of the m7GpppG cap analog. (C) Structure of the anti-reverse cap analog (ARCA), which has an additional 3' hydroxyl methylation compared to m7GpppG.
However, the distribution of tail sizes within a single enzymatic polyadenylation reaction varies much more than synthetic mRNAs with encoded poly(A) tails. A consensus of the optimal poly(A) tail length has not been found, but generally tails longer than 100 nucleotides are most effective.

**Addition of Regulatory Elements**

The flexible nature of the synthetic mRNAs allows for inclusion of elements that specifically alter its expression. The localization of these regulatory elements in the UTRs permits addition with minimal coding sequence perturbation. These elements can be tailored and combined to increase the complexity of the regulatory circuits and direct the expression of the synthetic mRNA in a more cell-specific or environmentally responsive manner.

**microRNA Switches**

Placing miRNA target site(s) in the 3' UTR of a reporter gene provides a simple way to validate the target site. This strategy has been expanded to confer cell-selectivity to plasmid and viral vectors by including target sites for miRNA that are highly expressed by cells that are not the intended target of the vector. The same strategy has also been applied to synthetic mRNAs, forming a miRNA switch. Studies utilizing miRNA switches have shown that despite the rapid translation of synthetic mRNA, silencing by endogenous miRNA is still effective. Furthermore, it was also discovered that a single target site at the 5' UTR is able to provide equivalent silencing to four target sites in the 3' UTR.
**Aptamers**

In addition to specific sequence recognition mediated by Watson-Crick base pairing, specific secondary structures, called aptamers, can be added to the UTR of synthetic mRNA that are recognized by endogenous proteins. Inclusion of these aptamer sequences can allow the construction of protein-responsive synthetic mRNAs that are repressed by the presence of the aptamer-binding protein. Aptamers that recognize small molecules, such as tetracycline or Hoechst dye, can also be inserted near the start codon to block translation in the presence of their ligand. No ON-switch aptamers have yet been reported, likely due to the stabilization of aptamer structure upon ligand binding impeding translational machinery.

**Gene Circuits**

Although the inclusion miRNA target sites and aptamers in synthetic mRNA only provides a means of reducing translation, trans-regulatory effectors can be used to invert these effects by constructing a gene circuit. The design of cell-, ligand-, and environmental-responsive gene circuits is a rapidly growing subject in the field of synthetic biology. Recent reports have demonstrated cell-specific expression of reporters by miRNA-mediated silencing of an aptamer binding protein in a gene circuit composed of only synthetic mRNA. Similar gene circuits have also been designed to be responsive to small molecules like tetracyclin.

Perhaps the closest analog to an ON-switch aptamer is ligand-responsive programmed ribosomal frameshifting (PRF), which is employed by viruses to induce a specific frameshift during translation resulting in different open reading frame (ORF).
PRF allows viruses to overlap multiple ORFs and reduces the size of the viral genome. Placing a ligand-sensing aptamer overlapping the end of the PRF sequence, the PRF is abolished in the presence of the ligand. Alternatively, introducing a hairpin structure between the PRF signal and the aptamer disrupts both sequences in the absence of the ligand. Binding of the ligand to the aptamer leads to destabilization of the hairpin and induces the PRF. Multiple PRF signals can be incorporated into a single synthetic mRNA, allowing for multiple inputs and outputs.

**In vitro Protein Complexing**

Recent work has shown that complexing synthetic mRNA with the eIF4E protein prior to encapsulation in a nanocarrier increased production. Part of this increased translation may be due to a measured increase in the stability of the mRNA-eIF4E complexes over uncomplexed mRNA. However, the enhancement by eIF4E was dependent on the polyamine used to form the nanocarrier. Further investigations are needed to test the applicability of *in vitro* protein complexing with other regulatory protein and nanocarrier systems.

**Circular mRNA**

Although eukaryotic mRNA is positioned in a nearly circular manner during translation, ribosomes must be recycled and reassembled to complete additional rounds of translation. Joining of the 5’ and 3’ ends of uncapped, untailed synthetic mRNA has been shown to produce circular mRNA. Translation of these circular mRNAs occurs in human cells even in the absence of an IRES sequence. Furthermore, the circular
mRNAs exhibit increased resistance to RNase degradation due to the lack of terminal nucleotides for exonuclease to attack\textsuperscript{154}. Recent work has demonstrated the efficacy of circularizing mRNA for increasing the production of recombinant proteins by removing the stop codon and introducing self-cleaving peptides\textsuperscript{155}. Other work has shown that fully complementary miRNA target sites can direct cleavage of circular RNA, which could be exploited to create circular versions miRNA switches to increase protein production\textsuperscript{156}.

**Self-replicating mRNAs**

The short lifetime of synthetic mRNAs necessitates repeated treatments to maintain protein expression. However, exploitation of alphaviral genomic RNA allows for the production of self-replicating synthetic mRNAs. The alphavirus family are plus-strand RNA viruses with genomes that encoded two polyproteins: the 5’ non-structural proteins (NSP) produced by cap-dependent translation and the 3’ structural proteins produced by translation from a subgenomic mRNA\textsuperscript{157}. The NSPs transcribe the minus-strand RNA necessary for genome replication and subgenomic mRNA transcription. The compartmentalization of the structural proteins into the subgenomic mRNA allows for their replacement with a gene of interest without altering the replicative capacity of the alphaviral genomic RNA. The resulting self-replicating mRNA produces very durable expression, far surpassing that regular synthetic mRNA and plasmid vectors. In ancillary experiments performed during the work reported in this dissertation, expression of a self-replicating green fluorescent protein (GFP) mRNA with a built-in puromycin resistance gene was observed in adherent-human embryonic kidney (Ad-HEK293) cells
out to three months (90 days) after transfection (Figure 2.4). These self-replicating mRNAs can also be regulated as part of a gene circuit

**Synthesis of synthetic mRNAs**

One of the benefits of synthetic mRNAs over other gene vectors is their ease of production. While it is possible to synthesize mRNAs base-by-base via nucleotide synthesizers, efficient production is limited to mRNAs with fewer than 100 bases due to the compounding effect of imperfect coupling efficiency. Therefore, synthetic mRNAs are usually generated by IVT from a DNA template. The resulting mRNA product must also be purified to remove the DNA template, reaction enzymes, free nucleotides, and potentially immunogenic dsRNA contaminants.

**Enzymatic synthesis by in vitro transcription**

IVT is typically performed using a T7 or SP6 bacteriophage RNA polymerase. The structure of these two polymerases is highly similar, but each polymerase requires a specific and short promoter for initiation of transcription. Transcription continues until the polymerases reach a terminator sequence. However, both T7 and SP6 polymerase exhibit a very high rate of readthrough transcription. This readthrough transcription is fundamental to establishing the stoichiometric ratio of the bacteriophage genes and is driven by the high processivity of the RNA polymerases. The processivity of the polymerases also enables IVT reactions to be scaled up to produce large quantities of synthetic mRNA.
Template Optimization for in vitro transcription

Both T7 and SP6 are DNA-dependent RNA polymerases, and therefore synthetic mRNA must first be constructed in a DNA template. Both plasmids and polymerase chain reaction (PCR) dsDNA products can be used for IVT. Because of the previously discussed readthrough transcription common to T7 and SP6, the use of an intact plasmid template leads to generation of a heterogenous pool of products. Products of a defined length are produced from plasmids that have been cut with a restriction enzyme immediately downstream of the synthetic mRNA sequence. However, a restriction enzyme that produces a blunt end or 5’ overhang should be used to prevent the polymerase from reinitiating on a transcript and producing larger than expected RNA \(^{161}\). If a PCR product is to be used as the template for IVT, gel purification after electrophoresis is recommend to ensure proper product size.

Optimization of the DNA template may be required for efficient IVT. The presence of T7 or SP6 terminator sequences in the template can lead to production of shorter than expected and heterogenous RNA products. The specificity of the terminator sequences enables their removal with only a few changes to the DNA template sequence. In addition, highly structured DNA elements like G-quadruplexes can block elongation of the RNA transcripts \(^{162}\). These structured regions can also be disrupted by altering only a few nucleotides.
Figure 2.4. Duration of self-replicating GFP mRNA expression in Ad-HEK293 cells.

Flow cytometry analysis of GFP expression in Ad-HEK293 cells after transfection with a self-replicating mRNA encoding GFP and a puromycin resistance gene. Cells were maintained in puromycin-supplemented medium for the duration of the experiment. Experiment was stopped on day 91 post-transfection.

**Synthetic mRNA Purification**

Purification of synthetic mRNA is necessary to ensure maximum efficacy and minimal immunogenicity. Many RNA purification kits are commercially available, but these kits can be replaced and supplemented with other techniques. The overall goal of these steps is the removal of template DNA, free nucleotides, reaction enzymes, and dsRNA products.

Ethanol, isopropanol, or a mixture of phenolic acid and chloroform are commonly used to concentrate nucleic acids and remove of protein contaminants. The inclusion of high concentrations of salts facilitates precipitation of the nucleic acids. Ammonium acetate is commonly used for purifying mRNA products from IVT as it is capable of
specifically precipitating RNA, leaving protein, DNA, and free nucleotides in solution. Lithium chloride is also suitable for mRNA purification but requires a higher concentration of mRNA for effective precipitation \(^{163}\).

**Elimination of double-stranded RNA**

Due to the immunogenicity of dsRNA, removal of dsRNA products may be required. Comprehensive removal of dsRNA can be accomplished by fractionation using high performance liquid chromatography (HPLC). HPLC purification also allows for removal of incorrectly sized ssRNA products if needed \(^{85}\). Recently, it has been shown that dsRNA can be specifically removed by centrifugation through a cellulose filled spin column, allowing for much more rapid purification without the need for specialized HPLC equipment \(^{164}\).

**Therapeutic Applications of synthetic mRNA**

The advantages of synthetic mRNA over plasmid or viral vectors, in conjunction with the recent advances in the increasing the efficacy and regulation of synthetic mRNA, have poised this promising class of biological drugs to revolutionize gene therapies. The rapid advances in the past decade have produced a plethora of biotechnology companies focused on the therapeutic application of synthetic mRNA. In addition, the initial successes seen so far have drawn the attention of traditional pharmaceutical companies which have begun investing in mRNA therapeutics. There are currently over 45 ongoing clinical trials involving delivery of synthetic mRNA to treat
a variety of diseases and disorders.

**Protein Replacement**

The most basic therapeutic application for synthetic mRNA is protein replacement. Initial studies utilizing synthetic mRNA were able to produce appreciable amounts of protein after injection in to animals\textsuperscript{165}. Synthetic mRNA was even shown to provide better results than recombinant protein therapies in a mouse model of Factor XI-deficient hemophilia B\textsuperscript{166,167}. However, only a few clinical trials involving delivery of synthetic mRNA for protein replacement are currently active.

A Phase II trial investigating the delivery of vascular endothelial growth factor A (VEGF-A) mRNA via epicardial injection during coronary bypass surgery (NCT03370887) is currently recruiting patients (Table 2.1). The preclinical studies using this strategy in a myocardial infarction model in rodents and swine has demonstrated a significant increase in revascularization of the infarct area and decreased fibrosis\textsuperscript{168}. Another clinical trial for VEGF mRNA is likely to be started to investing the efficacy in improving wound healing in diabetic individuals based on preclinical results in mice and successful completion of a Phase I safety study (NCT02935712)\textsuperscript{169}.

A Phase I/II clinical trial (NCT03375047) is also underway for the treatment of cystic fibrosis with nebulized synthetic mRNA. Preclinical results in mice have indicated that up to 55\% of normal chloride efflux could be restored by nebulization of cystic fibrosis transmembrane conductance regulator (CFTR) mRNA. Approval was recently given to assess the safety of multiple dosing of enrolled patients.
A similar Phase I/II clinical trial (NCT03767270) for treatment of ornithine transcarbamylase (OTC) deficiency, a hereditary disorder that leads to accumulation of ammonia in the blood after protein metabolism, has been submitted. Unlike the previous trials, NCT03767270 involves systemic administration of the synthetic mRNA to target the liver. However, the trial was placed on hold prior to patient enrollment until more preclinical data can be shown.

Vaccines

The properties of synthetic mRNA, particularly the rapid and transient expression of the encoded proteins, make it an ideal platform for vaccine development. Synthetic mRNA vaccines have shown promising preclinical results for a wide variety of infectious diseases such as: influenza, rabies, chikungunya, HIV, Ebola, and Toxoplasmosis gondii. The ability to quickly generate antigen-encoding synthetic mRNA accelerates the development of mRNA vaccines for emerging diseases. For example, the 2016 outbreak of Zika virus prompted the development of mRNA-based vaccines by two independent groups by early 2017. The optimal design of the synthetic mRNA used for vaccines remains to be determined. Self-replicating mRNAs are frequently used as they allow for durable expression and robust induction of antigen-specific antibodies. Synthetic mRNA vaccines can also be made self-adjuvanted by using unmodified mRNA that stimulates humoral immune responses via TLR7.

Clinical trials for synthetic mRNA vaccines against rabies virus (NCT03713086), metapneumovirus/parainfluenza (NCT03392389), cytomegalovirus (NCT03382405), chikungunya virus (NCT03829384), and influenza (NCT03345043) are currently
ongoing (Table 2.1). While preliminary results indicate that these treatments are safe, the protective effects of these vaccines have been found to vary significantly depending on the delivery method (i.e. needle or needle-free)\textsuperscript{175}.

Synthetic mRNA can also be delivered to \textit{ex vivo} to a patient’s dendritic cells, targeting them to the encoded antigen, before reinjection. Numerous clinical trials for HIV using mRNA-pulsed autologous dendritic cells (NCT00833781, NCT00672191, NCT01069809) found expected increases in antigen-specific T-cell response but observed no clinical benefit or decrease in viral load\textsuperscript{176,177}.

Cancer Vaccines

The flexibility of synthetic mRNA can also be utilized to encode tumor-specific antigens for vaccines against cancer. Unlike the prophylactic vaccines developed for infectious disease, cancer vaccines are designed to induce an immune response to tumor associated antigens (TAA) that are enriched on the surface of cancer cells or neoantigens that arise from somatic mutations. Immunotherapy allows for more specific targeting of cancer cells compared to traditional chemotherapies, greatly reducing the side effects common to anti-neoplastic therapeutics. The use of synthetic mRNA in cancer vaccines is almost exclusively in autologous dendritic cell therapies with numerous trials underway using this technique, including 3 Phase III trials (Table 2.1). Initial results of these trials have been promising and were bolstered by FDA approval of a peptide-based dendritic cell therapy (sipuleucel-T) in 2010.

Cellular Reprogramming
Reprogramming of somatic cells into pluripotent stems has many clinical applications, such as autologous stem cell therapies, drug screening, and disease modeling. Traditional generation of induced pluripotent stem cells (iPSC) relies on ectopic expression of the four Yamanaka factors: OCT4, KLF4, SOX2, and cMYC. Plasmid transfection and viral transduction are frequently used to produce iPSCs from a variety of somatic cell types. Synthetic mRNA is also capable of inducing cellular reprogramming but requires multiple transfections to maintain expression of the Yamanaka factors. However, incorporating all of the Yamanaka factors and a puromycin selection gene into a single self-replicating synthetic mRNA allowed for efficient iPSC generation with only a single treatment of the mRNA. Encoding all of the factors on a single transcript also ensures a consistent ratio of expression for each factor. Optimization of synthetic mRNA-mediated reprogramming has increased the efficiency to above 90%, allowing for iPSC generation from a very small number of primary cells. Synthetic mRNA can also be used to efficiently differentiate iPSCs into the desired somatic cell type.

**Genome Editing**

Correcting a genetic defect is the ultimate form of gene therapy. The recent discovery of the CRISPR/Cas9 nuclease system has caused a surge of genome editing research. The key advantage provided by Cas9 nucleases over older technologies such as Zinc-finger nucleases (ZFN) or transcription activator-like effector nucleases (TALEN) is the recognition of target sites by Watson-Crick base pairing via a guide RNA rather than direct recognition by the nuclease.
Various delivery strategies have been developed for all of the available gene editing systems. For clinical applications the duration of nuclease expression must be balanced by the need for sustained nuclease activity to ensure on-target correction and minimized expression to prevent off-target activity. There has been a recent shift toward delivery of synthetic mRNA encoding Cas9 rather viral or plasmid delivery. Gene circuits have also been designed that could be used to further control the expression and activity of Cas9 to reduce off-target effects.

Synthetic mRNA encoding Cas9 and IVT guide RNA have been shown to produce durable knockdown of a liver-specific gene in vivo following a single dose. Synthetic mRNA encoding a CCR5-targeting ZFN (SB-728) is currently in use in two clinical trials (NCT02500849, NCT02388594) aimed at establishing a HIV-resistant T cell population in patients undergoing retroviral therapies. However, gene correction requires a DNA repair template, which precludes the development of a synthetic RNA-only system. Despite this shortcoming, synthetic mRNA encoding nucleases could be used to reduce off-target effects in the ex vivo gene editing performed in a number of ongoing clinical trials (Table 2.1).
Table 2.1 Ongoing and recently completed clinical trials utilizing synthetic mRNA.

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### Table 2.1 (Continued)

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*ND: Not disclosed; PCV: Personalized Cancer Vaccine*
Mechanisms of Cellular Uptake

The cellular membrane provides a vital barrier between the highly regulated cytoplasm and the extracellular environment. While small molecules are able to pass through specialized channel proteins that span the cellular membrane, larger molecules like proteins and highly-charged molecules require active transport to enter the cytosol. This active transport often involves the formation of specialized vesicles called endosomes that encapsulate a small volume of the extracellular environment for further processing inside the cell.

Receptor-mediated Endocytosis

The selective cellular uptake of large molecules, such as growth factors or low-density lipoprotein (LDL), is mediated by binding to a cognate receptor. The receptor's intracellular domain engage adaptor proteins complexes that recruit clathrin triskelia. Interestingly, the binding of adaptor proteins is not dependent on ligand binding to the receptor. The most well studied adaptor protein is Adaptor protein 2 (AP2), which composed of heterotetramer of α, β2, μ2, and σ2 subunits. Upon binding to the cytosolic domains of receptor proteins, AP2 undergoes a conformational change stabilized by interaction with Phosphatidylinositol 4,5-bisphosphate (P(4,5)P2) lipids in the plasma membrane and exposes the clathrin-binding site on the β2 subunit \(^ {189,190}\). A single
clathrin triskelion consists of three interlocking spirals, each formed by a complex of a single heavy chain and light chain. Individual clathrin triskelia interact to form a lattice or cage anchored to the plasma membrane by AP2. However, both of these interactions are relatively unstable and allow for shuttling of clathrin to and from the lattice. Cargo binding to the receptors stabilizes the interaction of AP2 with the cytosolic domain and promotes retention of associated clathrin. Proximity of multiple AP2-bound receptors promotes the formation of a clathrin-coated pit (CCP), which has an average diameter of ~40 nm. CCP formation is driven by the intermolecular forces between clathrin triskelia that arrange themselves into a truncated icosahedron. As the CCP invaginates, the clathrin triskelia closest to the undeformed cell membrane assemble without binding to AP2, leading to AP2 enrichment at the bottom of the invagination \(^{191}\). While a CCP can spontaneously form de novo due to stoichiometric proximity of receptors, the duration of such CCP is much lower than those formed with ligand-bound receptors \(^{192}\).

Cleavage of the CCP to form a clathrin-coated vesicle (CCV) requires the pinching of the top of the CCP. The mechanism responsible for this are largely unknown but are dependent on the GTPase activity of dynamin. Inhibition of the GTPase activity of dynamin blocks endosomal constriction and CCV scission, resulting in accumulation of unconstricted or totally constricted CCP \(^{193}\). Once cleaved from the membrane, the clathrin coating of CCV is rapidly removed by heat-shock cognate protein (Hsc70) \(^{194}\). The uncoated vesicles are then trafficked to sorting endosomes to separate the receptors and the bound cargo.
Caveolae

Caveolae, like CCP, are invaginations in the cell membrane that can pinched off to form endocytic vesicles. However, caveolae also play a role in a variety of other cellular processes, such as mechanosensing, intercellular signaling, and lipid metabolism \(^{195-197}\). Caveolae are \(~70\) nm in diameter and characterized by the presence of caveolin proteins embedded in the lipid bilayer. Caveolin-1 (CAV-1) was the first caveolin protein identified and has been shown to enrich caveolae with cholesterol though direct binding at the C-terminus. CAV-1 enriched membrane domains are stabilized by the binding of Cavin1, which also interacts with PI(4,5)P\(_2\). The binding of Cavin1 is thought to induce the invagination of caveolae, though exact mechanisms remain unclear \(^{198}\). Scission of caveolae from the plasma membrane, like CCV, is driven by the GTPase activity of dynamin \(^{199}\). The endocytic caveolae vesicle then fuses with early endosomes for further processing, although there have been reports of clusters of endocytic caveolae vesicles distinct from endosomes \(^{200}\).

Macropinocytosis

Macropinocytosis, encompasses all endocytic processes that are clathrin- and caveolae-independent and occurs in all cell types. The formation of invaginations during macropinocytosis is driven by the formation of a distinct ring of filamentous actin (F-actin) \(^{201}\). These contractile F-actin rings can arise spontaneously or can be formed by closure of circular membrane ruffles \(^{202,203}\). The expansion of the F-actin ring outward from the forms the overlying plasma membrane into cup, eventually creating a tight neck similar to that of other endocytic vesicles. However, the mechanism of
macropinosome scission is not currently known, but it is known to occur independent of dynamin activity\textsuperscript{204}. Once fully cleaved from the membrane, macropinosomes enter the endosomal trafficking pathway along with CCV and endocytic caveolar vesicles.

**Maturation of Endocytic Vesicles**

Most endocytic vesicles are processed through the endosomal pathway after membrane scission. This pathway serves a hub of intracellular trafficking and can sort endocytosed particles for degradation, cytosolic release, or recycling to the plasma membrane. The endpoint of endosomal trafficking is largely determined by binding of various proteins markers, particularly members of the Rab GTPase family, and conversion of phospholipids in the endosomal membrane during the process of endosomal maturation.

**Early Endosomes**

The first stage of endosomal trafficking is characterized by fusion of multiple vesicles of the same type to form an early endosome. The early endosome serves as both a buffer between nascent vesicles and the primary cargo sorting compartment. The amount of membrane, number of receptors, and enclosed volume of the incoming vesicles necessitates rapid recycling from the early endosome back to the plasma membrane by Rab4. The release of cargo from the cognate receptor is facilitated by the decreased pH (~6.5) in the early endosome, which is driven by the vacuolar H\textsuperscript+-ATPase (V-ATPase). The positive charge of the transported protons is offset by the presence of
vacuolar chloride channels, minimizing the electrochemical gradient across the endosomal membrane \(^{205}\).

The early endosome is characterized by the accumulation of Rab5 on the outside of the endosomal membrane. Rab5 recruits phosphatidylinositol 3-kinase to the endosomal membrane which converts phosphatidylinositol (PtdIns) to PtdIns(3)P (PI(3)P). PI(3)P is recognized by Endosomal Sorting Complexes Required for Transport (ESCRT)-0, which also binds to the intracellular domains of endocytosed receptors marked for degradation. Subsequent recruitment of ESCRT-1, 2, and 3 localizes the marked receptors into invaginations within the early endosome. Inward deformation of the endosomal membrane by the ESCRTs leads to the formation of an intraluminal vesicle \(^{206}\).

**Late Endosomes/Lysosomes**

The maturation of early endosomes to late endosomes is marked by a switch in the binding of Rab5 to Rab7. Recent work shows that this change is mediated by recruitment of Rab7 by the binding of SAND-1 to Rab5 \(^{207}\). In addition, the PI(3)P in the endosomal membrane can be further phosphorylated to PI(3,5)P\(_2\) by PIKfyve, and accumulation of PI(3,5)P\(_2\) reduces further binding of nascent endocytic vesicles to the maturing endosome \(^{208}\). During the maturation process the endosome continues to acidify, reaching a pH of \(\sim 5.5\) in the late endosome.

The maturing endosome is transported along the cytoskeleton toward the nucleus. Along the way vesicles containing various lysosomal acid hydrolases fuse with the endosome and being degrading the endosomal cargo. This degradation can be
accelerated by fusion of the endosome with established secondary lysosomes\textsuperscript{209}. After degradation, the excess membrane of the lysosome is recycled back to the Golgi thereby reforming the secondary lysosome\textsuperscript{210}.

**mRNA Delivery Methods**

The delivery of synthetic mRNA is the largest barrier to wider clinical applications. There are two major problems that must be resolved: delivery of synthetic mRNA to the target organ/tissue/cells and endosomal escape. To overcome these obstacles, a variety of methods have been developed to transfect synthetic mRNA into cells.

**Physical Transfection Methods**

The cellular membrane prevents passive diffusion of mRNA molecules into cells due to their large size and high negative charge. Because synthetic mRNA need only to access the cytoplasm to produce their effect, bypassing the cell membrane can be an effective method of delivery.

*Electroporation*

Electroporation is widely used in the laboratory setting to deliver DNA into cells. The generation of a strong local electric field forms pores in the cellular membrane which allows large molecules to diffuse into the cell. The efficiency of *in vitro* electroration is very high due the ability to concentrate the recipient cells and payload nucleic acid in a very small volume. However, the stress of the electroration results in
a high rate of cell death, necessitating the use of large numbers of cells to recover a viable population of transfected cells.

In addition to *in vitro* applications, electroporation has also been demonstrated to effectively deliver nucleic acid payloads *in vivo*. The focal application of the electric field allows for very precise targeting of payload nucleic acids. However, the small size of the transfected area reduces the feasibility of such a transfection method in larger organisms and would necessitate multiple applications to cover a significant area.

Electroporation has been widely employed in *ex vivo* transfection of autologous cells. Many clinical trials for cancer vaccines employ dendritic cells that are pulsed with mRNA via electroporation (**Table 2.1**). Electroporation is well suited for such therapies because the cells collected from the patient can be expanded before and after transfection.

*Biolistic Delivery*

An alternative to electroporation for topical delivery of nucleic acids is the use a biolistic delivery system, commonly referred to as a “gene gun”. This method of transfection utilizes nucleic acids complexed to inert metal, typically gold, nanoparticles that are accelerated high speeds by compressed gas. The fast-moving nanoparticles are capable of penetrating through cell membranes.

Biolistic delivery is commonly employed in agriculture research. Like *in vivo* electroporation, biolistic delivery is well suited for applications that need only a localized expression of the delivered nucleic acid to produce the desired effect. Biolistic delivery of DNA vaccines have shown some efficacy, but the immune response to these
vaccines differs from those delivered by intramuscular injection. Topical (<1 mm) transfection of mRNA into the skin or kidney has also been demonstrated using a gene gun in a rat model, along with a potential application in accelerating wound healing.

**Microinjection**

Synthetic mRNA can also be directly injected into the cytoplasm of a cell using a micropipette. Due to the intensive labor and low throughput of this technique, it is typically used to deliver synthetic mRNA encoding Cas9 to perform gene editing in zygotes.

**Nanoparticle Delivery Platforms**

While mechanical transfection methods are sufficient for localized transfection of synthetic mRNA, systemic delivery poses additional challenges. Firstly, the stability of mRNA in the bloodstream or extracellular interstitium is extremely low due to the presence of extracellular RNAse. Therefore, it is necessary to shield the synthetic mRNA from the surrounding environment in a nanoparticle by encapsulating the payload mRNA away from the environment or by winding the mRNA within the nanoparticle structure. Secondly, the nanoparticles and their synthetic mRNA payload must be capable of entering cells. Unlike mechanical transfection methods, nanoparticles must be taken up by cells via endocytosis. Efficient uptake of nanoparticles by cells is dependent on both the hydrodynamic diameter and the surface charge of the nanoparticle. Internalization can also be affected by recognition by cell-surface receptors. Lastly, once the nanoparticles are endocytosed, the synthetic mRNA payload must be released to the cytoplasm. This requires both disassembly of
the nanoparticle and escape from the endosome. Endosomal escape in particular remains a significant challenge for most of the synthetic mRNA delivery platforms currently in use, with typical endosomal escape rates averaging approximately 2% \(^{218}\). Nanoparticle design must also consider the balance between the stability of nanoparticles in the extracellular space and the ability to release the payload from the mRNA once inside of cells.

The similarity of synthetic mRNA and siRNA has allowed the results from the more mature field of siRNA therapeutics to guide the development of mRNA delivery platforms. There are two classes of nanoparticles that are the focus of intense pre-clinical research, lipid and polymer-based nanoparticles. Both systems have received approval from the US Food and Drug Administration (FDA), though only one lipid nanoparticle has been approved for delivery of a therapeutic RNA (Patisiran). However, the variety of compositions possible when designing these delivery systems has begun to blur the lines between these categories.

**Lipid Nanoparticles**

Lipid nanoparticles (LNP) are the most well-studied delivery platform for synthetic mRNA delivery. The structure of LNP is defined by the formation of a lipid bilayer around the nucleic acid containing core. Altering the composition of the bilayer is the focus of most LNP development strategies. The earliest LNP were formed from bilayers containing a mixture of cationic and neutral lipids, N-[1-(2,3-Dioleyloxy)propyl]N,N,N,N-trimethylammonium Chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)
The positively charged head of the cationic lipid allows electrostatic interaction with the negatively charged backbone of nucleic acids and promotes efficient encapsulation. The efficacy of these LNPs has been increased by adding stabilizing and shielding components to the lipid bilayer. Cholesterol is also incorporated during LNP formation to increase the stability of the lipid bilayer. Helper lipids bearing polyethylene glycol (PEG)-conjugated lipids are also frequently used to form the outer layer of the LNP. The PEGylated lipids play a key role in the determination of LNP size due to steric stabilization by the PEGylated head groups.

Due to undesirable interaction with negatively charged serum proteins and subsequently rapid clearance the use of cationic lipids for in vivo applications is very limited. To overcome these effects, permanently cationic lipids have largely been replaced with ionizable lipids that are cationic only in acidic environments. As a consequence of this inducible charging, formation of LNP with ionizable lipids must be carried out in an acidic environment. To facilitate efficient packaging of the payload nucleic acid, LNPs are typically constructed using microfluidic mixing. This technique allows for precise control over the ratio of ionizable lipid, helper lipids, and payload, as well as the timing of the component addition. A wide variety of ionizable lipids have been developed, but the details the composition of LNP in clinical development by various biotechnology companies are proprietary information.

The positive charge of LNP facilitates their interaction with the negatively charged membranes of cells, which can facilitate their uptake by endocytosis. Once in contact with a cellular membrane, the cationic lipids or protonated ionizable lipids are induced to flip between the membranes by anionic lipids in the cellular phospholipid
bilayer. The electrostatic interaction of the cationic and anionic lipid head groups, alters the geometry of the hydrophilic tails, and induces the adoption of hexagonal structure that has been shown to destabilize the endosomes. However, uptake of LNP induces a variety of cytotoxic effects, including vacuolization of the cell and reduced proliferation.

**Polymeric Nanoparticles and Nanomicelles**

Nucleic acids can also be packaged by electrostatic interaction with polymers. Polyethylenimine (PEI) is the prototypical polymeric transfection reagent and has been widely used for *in vitro* transfection of DNA in the laboratory settings. PEI can be produced with or without branching and at various molecular weights. The size and transfectability of PEI nanoparticles is dependent on the structure of the PEI and the conditions of nanoparticle formation. In particular, increasing the molecular weight of PEI leads to formation of smaller nanoparticles due to the increased positive charge carried by the PEI polymer. In addition, branched PEI nanoparticles are more transfective *in vitro* while linear PEI nanoparticles are more transfective *in vivo*. Regardless of the structure of the PEI used, the resulting nanoparticles are formed by condensing of nucleic acids rather than encapsulation.

In contrast, the encapsulation of nucleic acids into a nanomicelle can be achieved using amphiphilic co-polymers that contain both hydrophilic hydrophobic domains, such as PEG-polyaspartamide. Endosomal escape of polymeric nanoparticles and nanomicelles is due to osmotic swelling driven by “proton sponging”, which draws additional chloride ions and subsequently water into the acidifying
endosome. One drawback of polymeric nanoparticles and nanomicelles is the persistence of the polymer after payload delivery, whose accumulation can induce apoptosis in cells. However, degradable polymers or derivatives can be used to reduce the accumulation and associated toxicity. Recent work has demonstrated the utility of encapsulating a polymeric nanoparticle in a lipid bilayer to form a hybrid nanoparticle.

Cell Penetrating Peptides

Cell penetrating peptides (CPP) have received little attention as a delivery platform for synthetic mRNA. CPP are diverse class of proteins and peptides that possess the ability to translocate across or disrupt the integrity of phospholipid bilayers. For cargo delivery, CPP are typically covalently linked to their cargo. Such a linkage is suitable delivery of siRNA and miRNA antagonists since these systems utilize an inactive passenger strand of RNA, but covalent linkage to an mRNA would likely disrupt translation.

Cationic and amphipathic CPP have been shown to form non-covalent electrostatic interactions with the negatively-charged phosphodiester backbone of RNA and DNA which can enable their delivery into cells. Most of these CPP are permanently charged due to the presence of arginine and lysine residues, which stabilizes their interaction with nucleic acids. Excessive stabilization can prevent CPP from effectively delivering their cargo due to slow disassembly of the CPP-nucleic acid complex and therefore poor endosomal escape, necessitating the use of endosomolytics such as chloroquine.
Alternatively, CPP that are not designed to interact with nucleic acids have been incorporated into lipid or polymeric nanoparticles to increase their endosomal escape. Melittin, a pore-forming CPP that is the primary component of bee venom, has been shown to be particularly effective when employed in such a way \(^{234}\). Melittin has also been explored as a cytotoxic agent for treating cancer via delivery in perfluorocarbon nanoparticles \(^{235,236}\). The strong lytic activity of melittin can produce significant cell death, but the inclusion of pH-sensitive protecting groups that limit melittin’s activity until it reaches the endosome ameliorated this cytotoxicity \(^{237}\). This pH-sensitive melittin was employed as an endosomal escape adjuvant by Arrowhead Pharmaceuticals in a clinical trial for an siRNA treatment for Hepatitis B infection (NCT02452528).

Unfortunately, even the pH-sensitive form of melittin proved hepatotoxic at high doses, and the death of non-human primates in pre-clinical studies prompted the FDA to halt the ongoing clinical trial. Recently, Arrowhead Pharmaceuticals has resumed clinical trials using this system via subcutaneous delivery (NCT03365947).

Recent work has shown that truncated forms of melittin exhibit reduced lytic activity \(^{238}\). These melittin-derived peptides could insert into the membrane of liposomes and perfluorocarbon nanoparticles to act as an anchor for surface conjugates, such VCAM-1 targeting ligands \(^{239}\). Further modification of these peptides that added a pH-sensitive cationic charge on the C-terminal end of the peptide (p5RHH) enabled the electrostatic conjugation of the peptide to an siRNA cargo \(^{240}\). The inclusion of pH-sensitive histidine residues allowed the p5RHH-siRNA nanoparticles to disassemble in acidifying endosomes, resulting in a high localized concentration of the p5RHH and subsequent endosomolysis \(^{241}\). p5RHH-siRNA nanoparticles have been reported to
effectively deliver their siRNA payloads to inflammatory macrophages in models of arthritis. In addition, p5RHH-siRNA nanoparticles have been shown to accumulate in areas of disrupted endothelium, such as tumors and atherosclerotic plaques. Adaptation of the p5RHH nanoparticle system to deliver synthetic mRNA could enable the development of mRNA therapeutics for these diseases.

**In vivo distribution of Nanoparticles**

In addition to the challenges faced in the uptake and endosomal escape synthetic mRNA-loaded nanoparticles, targeting specific organs remains an unsolved challenge. Furthermore, the short duration of expression from synthetic mRNA is exacerbated to the rapid clearance of nanoparticles due to opsonization by serum proteins. However, alterations in the composition of the nanoparticle surface and composition begun to show improvements in these areas.

**Adsorption of Serum Proteins**

Initial studies with cationic LNP found that avidly bound by serum proteins, primarily due to the strong positive surface charge of the LNP. This adsorption created a protein corona around the LNP, increasing its diameter and imparting a negative surface charge. The primary serum components responsible were identified as albumin, high and low density lipoproteins (HDL, LDL), and macroglobulin. Similar accumulation of serum proteins was also observed when using cationic polymeric nanoparticles. The inclusion of PEGylated lipids or the use of PEG-copolymer blocks was found to significantly reduce the amount of opsonization and extend the circulation
time of the nanoparticles and is now used in the majority of nanoparticle formulations. As previously discussed, the binding of serum proteins to LNP was also reduced by utilizing ionizable lipids.

### Nanoparticle Clearance

The size of nanoparticles plays a large role in their clearance from the circulation. The smallest nanoparticles, up to 5 nm in diameter, are removed from the blood by glomerular filtration in the kidney. However, virtually any opsonization by serum proteins will increase the effective diameter of the nanoparticle above this threshold and prevent renal clearance. Larger particles are instead removed from circulation by uptake and degradation in the liver.

### Organ Sequestration

Given the relatively large size of LNP and polymeric nanoparticles, accumulation of particles is typically observed almost exclusively in the liver after systemic administration. The accumulated nanoparticles are avidly endocytosed by hepatocytes due to opsonization by apolipoprotein E (ApoE). Because of the preferential uptake in the liver, most applications for systemically administered nucleic acid therapies either target liver-specific disorders, as in the case of siRNA, or utilize the liver as a biosynthetic depot. In addition, the accumulation of a protein corona on the surface of non-PEGylated nanoparticles significantly increases their uptake by cells of the mononuclear phagocyte system (MPS).
Alteration of the components used to build LNP or polymeric nanoparticles can confer a degree of tissue specificity. For example, one group demonstrated a ionizable LNP that resulted in nearly exclusive expression of the payload luciferase mRNA in the immune cells of the spleen despite over a 10-fold higher uptake of particles by the liver. Another group has developed a LNP that preferentially expressed luciferase in the lungs. Further development of these nanoparticle systems may involve the addition of targeting moieties to the surface of the nanoparticles, a strategy that has been demonstrated for siRNA-loaded polymeric nanoparticles.

Nanoparticle-associated Toxicity

For clinical applications, the safety and toxicity of nanoparticles and their components must be carefully examined. The cationic lipids commonly employed in early LNP were found to produce unacceptable levels of immunogenicity and hepatotoxicity due to induction of inflammatory cytokines and activation of TLR. However, the switch to ionizable lipid formulations has virtually eliminated these associated toxicities. The PEG-shielding commonly used in nanoparticles may prove to be a double-edged sword as anti-PEG antibodies have been detected in over 20% of blood samples without prior exposure to PEGylated drugs. These antibodies can significantly accelerate the clearance of PEGylated nanoparticles, reduce their efficiency, and potentially lead to adverse immune responses due to activation of the complement system.
Gap in Knowledge and Purpose of this Dissertation

Synthetic mRNA as a therapeutic modality has been greatly advanced in recent years. The discovery of modified nucleotides, in particular, has reignited interest in the use of synthetic mRNA over other gene vectors due to the reduction of innate immunogenicity. The incorporation of target sites for endogenous regulators, such as miRNA, and the rational design of gene circuits hold great promise for enabling tissue-, cell-, or even environmental-responsive therapeutics. However, changes in the efficiency of these regulatory elements due to incorporation of modified nucleotides, which have been shown to alter mRNA structure and decoding, have not been examined. To address this gap in knowledge, we transcribed synthetic miRNA-switches with a variety of commonly used modified nucleotides. We examined the effects of the nucleotides on the regulation of translation by various species of miRNA. In addition, we also investigated the effect of sequence context and target site complementarity. We found that these modified nucleotides can indeed reduce the silencing of transcripts by miRNA, but this loss of regulation can be ameliorated by placing the miRNA target site in the 5’ UTR of the transcript.

The delivery of synthetic mRNA remains the largest barrier to the widespread application of this promising technology. Due to the sensitivity of mRNA to degradation by extracellular nucleases, synthetic mRNA must be packaged in protective nanoparticles. The size and charge of these nanoparticles drives their accumulation in the liver and uptake by cells of the mononuclear phagocyte system. While uptake by these cells can be avoided by minimizing the adsorption of serum proteins, delivering synthetic mRNA to other organs has proven challenging. Entrapment of mRNA-loaded
nanoparticles in the endosome after uptake also reduces the potency of mRNA therapeutics. Despite the rapid advancement of mRNA therapeutics, further work is necessary to overcome the remaining challenges to widespread clinical applications. While altering the constituents of the commonly used LNP and polymeric nanoparticles has yielded some improvements, investigation of novel mRNA delivery platforms is lacking. We investigated a small cell-penetrating peptide, called p5RHH, that has a cationic tail and is capable of spontaneously forming transfective nanoparticles with mRNA. We showed that these nanoparticles are avidly endocytosed by cells, and that the endosomal release of the payload mRNA is highly efficient. We also demonstrated that these nanoparticles exhibit a high degree of RNase resistance and minimal cytotoxicity. Finally, we found that systemic administration of these nanoparticles led to robust expression of a fluorescent reporter in atherosclerotic plaques of ApoE−/− mice, but produced no detectable expression in liver, spleen, lungs, or kidneys.
Chapter 4

Materials and Methods

Ethical Approvals

All animal experiments were approved by the IACUC of the University of South Florida (protocol #4197).

Cell Culture

B16F10 (ATCC CRL-6475), AD-HEK293 (Stratagene #240085), and HeLa (ATCC CCL-2) cells were cultured in DMEM with 10% FBS. HUVEC and VSMC (Lonza) were cultured in EGM-2 Endothelial Cell Growth Media or SMGM-2 Smooth Muscle Cell Growth Media (Lonza), respectively. All cells were maintained in sub-confluent densities to allow cell division throughout the course of the experiments. Experiments using HUVEC and VSMC were carried out in cells between 3 to 7 passages.

mRNA Template Construction

Green fluorescent protein (GFP) constructs for the generation of in vitro transcription were designed using the pLL3.7 plasmid, a gift from Luk Parijs (Addgene plasmid # 11795). The 3’-UTR of human β-globin (132 bp) was amplified using HeLa genomic DNA as template with forward and reverse primer oligonucleotides #1 and #2 (Table 4.1). The β-globin 3’-UTR PCR product and the pLL3.7 plasmid were then EcoRI digested and ligated together to complete the construct. BamHI, PacI, and SphI
restriction sites were included upstream of the β-globin 3'-UTR in oligonucleotide #1 to facilitate the insertion of miRNA target sites at the 3' UTR.

To construct GFP miRNA switches with one or two copies of the fully complementary target sites for miR-126-3p (GFP-1x126TS or GFP-2x-126TS), we annealed oligonucleotides #3-4 or oligonucleotides #5-6, respectively, and ligated them into the plasmid using the BamHI restriction site added to the GFP 3'-UTR (Table 4.1). To construct GFP with three or four copies of the fully complementary target sites for miR-126-3p at the 3'-UTR, we annealed oligonucleotides #7-8, containing two copies of miR-126-3p fully complementary target sites, and ligated them into the GFP-1x126TS or GFP-2x126TS plasmids using the PacI restriction site (Table 4.1). Likewise, to construct GFP with two miR-126-3p seed target sites (GFP-2x126seed) at the 3'-UTR, we annealed oligonucleotides #9-10 that contained two copies of regions complementary to the seed sequence of miR-126-3p and introduced them into the GFP plasmid using the BamHI restriction site. The same strategy was used to construct GFP-4x21TS, GFP-4x145TS and GFP-4x122TS plasmids by inserting four target sites for miR-21-5p (oligonucleotides #11-14), miR-145-5p (oligonucleotides #15-18) or miR-122-5p (oligonucleotides #19-22) respectively, using the BamHI and PacI sites (Table 4.1).
Table 4.1. List of oligonucleotides used to generate plasmids and templates for in vitro transcription of synthetic mRNA.

<table>
<thead>
<tr>
<th>Construction of plasmids</th>
<th>Forward/Reverse</th>
<th>Oligonucleotide sequence</th>
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</thead>
<tbody>
<tr>
<td>1 EcoRI-BamHI-Pacl-SphI-βglobin 3’UTR</td>
<td>Forward</td>
<td>5’-ATTA-gaatc-ggatc-nttaaa-gcatgc-GCTCGCTTCTTTGCTGTCCAAAATTCTA</td>
</tr>
<tr>
<td>2 EcoRI-βglobin 3’UTR</td>
<td>Reverse</td>
<td>5’-ATTA-gaatc-GCAATGAAAAATATGGTTTTTTTATAGCCAGAATCCAGAT</td>
</tr>
<tr>
<td>3 BamHI-1x126TS-BamHI</td>
<td>Forward</td>
<td>5’-gatcc-CGACATTATTACTACGGTACGA-g</td>
</tr>
<tr>
<td>4 BamHI-1x126TS-BamHI</td>
<td>Reverse</td>
<td>5’-gatcc-TCGACCGTGAGTAATAATGCG-g</td>
</tr>
<tr>
<td>5 BamHI-2x126TS-BamHI</td>
<td>Forward</td>
<td>5’-gatcc-CGACATTATTACTACGGTACGA-g</td>
</tr>
<tr>
<td>6 BamHI-2x126TS-BamHI</td>
<td>Reverse</td>
<td>5’-gatcc-TCGACCGTGAGTAATAATGCG-g</td>
</tr>
<tr>
<td>7 Pacl-2x126TS-Pacl</td>
<td>Forward</td>
<td>5’-ttaa-CGACATTATTACTACGGTACGA-g</td>
</tr>
<tr>
<td>8 Pacl-2x126TS-Pacl</td>
<td>Reverse</td>
<td>5’-ttaa-TCGACCGTGAGTAATAATGCG-g</td>
</tr>
<tr>
<td>9 BamHI-2x126seed-BamHI</td>
<td>Forward</td>
<td>5’-gatcc-TCGACCGTGAGTAATAATGCG-g</td>
</tr>
<tr>
<td>10 BamHI-2x126seed-BamHI</td>
<td>Reverse</td>
<td>5’-gatcc-TCGACCGTGAGTAATAATGCG-g</td>
</tr>
<tr>
<td>11 BamHI-2x21TS-BamHI</td>
<td>Forward</td>
<td>5’-gatcc-TCAACATCAGTCTGATAAGCTAgatcgatcTCAACATCAGTCTGATAAGCTA-g</td>
</tr>
<tr>
<td>12 BamHI-2x21TS-BamHI</td>
<td>Reverse</td>
<td>5’-gatcc-TACGGTACCTTACATTTAGTTAAATAAA-g</td>
</tr>
<tr>
<td>13 Pacl-2x21TS-Pacl</td>
<td>Forward</td>
<td>5’-ttaa-TCAACATCAGTCTGATAAGCTAgatcgatcTCAACATCAGTCTGATAAGCTA-g</td>
</tr>
<tr>
<td>14 Pacl-2x21TS-Pacl</td>
<td>Reverse</td>
<td>5’-ttaa-TCAACATCAGTCTGATAAGCTAgatcgatcTCAACATCAGTCTGATAAGCTA-g</td>
</tr>
<tr>
<td>15 BamHI-2x145TS-BamHI</td>
<td>Forward</td>
<td>5’-gatcc-AGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatcgatcAGGGATTCCTTGCGAAAGATGGCAGatc...</td>
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<tr>
<td>22</td>
<td>PacI-2x122TS-Pacl</td>
<td>Reverse</td>
</tr>
<tr>
<td>23</td>
<td>XbaI-βglobin 3’UTR</td>
<td>Forward</td>
</tr>
<tr>
<td>24</td>
<td>XbaI-βglobin 3’UTR</td>
<td>Reverse</td>
</tr>
<tr>
<td>25</td>
<td>Nhel-Luc</td>
<td>Forward</td>
</tr>
<tr>
<td>26</td>
<td>BamHI-Luc</td>
<td>Reverse</td>
</tr>
<tr>
<td>27</td>
<td>NotI-βglobin 3’UTR</td>
<td>Reverse</td>
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</tbody>
</table>

Table 4.1 (Continued)

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<tbody>
<tr>
<td>28</td>
<td>T7-βglobin 5’ UTR-GFP</td>
<td>Forward</td>
</tr>
<tr>
<td>29</td>
<td>T7-βglobin 5’UTR-nIRFP</td>
<td>Forward</td>
</tr>
<tr>
<td>30</td>
<td>T7-βglobin 5’UTR-Luc</td>
<td>Forward</td>
</tr>
<tr>
<td>31</td>
<td>βglobin 3’UTR</td>
<td>Reverse</td>
</tr>
<tr>
<td>32</td>
<td>T7-βglobin 5’UTR-1x126TS- GFP</td>
<td>Forward</td>
</tr>
</tbody>
</table>
Near infrared fluorescent protein 670 (niRFP) constructs were constructed in a similar manner using forward and reverse primer oligonucleotides #23 and #24 to clone the β-globin 3'-UTR from HeLa genomic DNA, followed by XbaI digestion and ligation of the PCR product and the piRFP-N plasmid, a gift from Vladislav Verkhusha (Addgene plasmid # 45457) (Table 4.1). Firefly Luciferase (Luc) constructs were created by cloning the luciferase coding sequence out of pGL2 Basic (Promega) using forward and reverse primer oligonucleotides #25 and #26 (Table 4.1). The Luc PCR product and the pLL3.7 plasmid with the β-globin 3'-UTR were digested with Nhel and BamHI, removing GFP from the pLL3.7 plasmid. The Luc coding sequences was then ligated into the pLL3.7 to form the final construct. The β-globin 3'-UTR was added to the 3' end of GFP in pT7-VEE-GFP, a gift from Steven Dowdy (Addgene plasmid #58977), after PCR with primers #23 and #27 by double digestion with XbaI and NotI (Table 4.1).

**in vitro Transcription**

For **in vitro** transcription, templates were generated by PCR using forward primers #28, #29, or #30 (containing a T7 promoter, 5'-UTR of human β-globin and the first ~20 bases of GFP, niRFP or Luc, respectively) and reverse primer #31 (starting at the end of the human β-globin 3'-UTR) (Table 4.1). Templates for the GFP miRNA switch with the miR-126-3p target in the 5' UTR were generated by using forward primer #32 and reverse primer #31 (Table 4.1). VEE-GFP template was generated by linearizing the pT7-VEE-GFP-βglobin plasmid with MluI.

In vitro transcription was performed, per the manufacturer’s protocol, using the HiScribe T7 High Yield RNA synthesis kit (NEB) followed by ammonium acetate.
precipitation as previously described. Pseudouridine-5'-Triphosphate (TriLink Biotechnologies, N-1019), 5-Methylcytidine-5'-Triphosphate (TriLink Biotechnologies, N-1014), N¹-methylpseudouridine-5'-Triphosphate (TriLink Biotechnologies, N-1081), and 2-Thiouridine-5'-Triphosphate (TriLink Biotechnologies, N-1032) were substituted during IVT to generate modified mRNA. The RNA was then capped using the ScriptCap™ m⁷G Capping System (CellScript) and tailed using the A-Plus™ Poly(A) Polymerase Tailing Kit (CellScript). The addition of >100 nt poly(A) tail was confirmed by electrophoresis on a 1% agarose gel. Following poly(A)-tailing, the mRNA was precipitated using 1x volume of 5M ammonium acetate, re-suspended in DNase/RNase-free water, quantified by spectrophotometry, and stored at -80 °C until use.

Preparation of p5RHH

p5RHH peptides were synthesized by Genscript (Piscataway, NJ, USA), dissolved at 5 mM in DNase/RNase-free water, and stored at -80 °C until use.

Formation and characterization of p5RHH-mRNA nanoparticles

The synthetic mRNA and p5RHH peptides were diluted to 70 ng/µL and 400 µM, respectively, in DNase/RNase-free water. The mRNA and p5RHH were then combined at a 1:1 volume ratio in 8 volumes of OptiMEM (Gibco) and mixed briefly. The solution was incubated at 37 °C for 40 minutes prior to use. To visualize p5RHH and mRNA binding a gel shift assay was performed on Cy5-labeled GFP mRNA complexed with increasing amounts of p5RHH. The resulting products were visualized by gel electrophoresis on an RNase-free 1% agarose gel.
To determine the size of the p5RHH-mRNA nanoparticles, a 30 µL volume of p5RHH-mRNA nanoparticle solution was pipetted onto a 200 nm polycarbonate track etched membrane, air dried, sputter coated with gold/palladium, and imaged using a JSM6490 Scanning Electron Microscope (JEOL).

mRNA and miRNA mimic Transfection

Cells were plated in 24-well plates (AD-HEK293, HeLa, VSMC: 5x10^4/well; HUVEC: 1x10^5/well) on the day prior to the transfection with miRIDIAN miRNA mimics (GE Dharmaco), miRNA mimics were transfected at a final concentration of 200nM using DharmaFECT #4 Transfection Reagent (Dharmacon). Lipofectamine® 2000 (Thermo Fisher Scientific) was used for mRNA transfections. Media was changed after four hours to remove transfection reagent from HUVEC, VSMC, and HeLa cells. Cells were collected 24 hours following mRNA transfection.

Cells were plated in 48-well plates at 15,000 cells/well on the day prior to transfection with the p5RHH-mRNA nanoparticles. Cells were transfected by replacing the cell culture medium with the p5RHH-mRNA nanoparticle solution supplemented with fresh culture medium to a final volume of 100 µL per well. Bafilomycin [1 µM] and chloroquine [50 µM] were added to the transfection media prior to addition to the wells.

For RNase protection assays 350 ng of GFP mRNA was complexed with 2.0 nmol of p5RHH prior to addition 0.25 µg RNaseA or vehicle control to the nanoparticle mixture and incubation at 37 °C for 20 minutes. An equal amount of uncomplexed GFP mRNA was also subjected to RNaseA treatment before nanoparticle formation. The
resulting products were added to B16F10 cells, and the percent of GFP positive cells were determined by flow cytometry after 24 hours.

**Flow Cytometry**

Prior to analysis by flow cytometry, cells were trypsinized, washed and resuspended with PBS. p5RHH-mRNA nanoparticle uptake and transfection efficiency were determined using a FACSCanto II (BD Bioscience). To assess cytotoxicity, propidium iodide was added to the resuspended cells 1 minute prior to analysis.

**Western Blotting**

Total protein lysates were fractionated by SDS-PAGE as previously described, transferred to nitrocellulose membranes, and blocked with Odyssey blocking buffer for 1 hour at room temperature. Membranes were probed overnight with rabbit anti-GFP (Invitrogen A-6455, 1:1000) and rabbit anti-α-tubulin (Cell Signaling Technology #2144) or rabbit anti-GFP (Life Technologies A-6455, 1:1000) primary antibodies followed by donkey anti-rabbit IgG IRDye680 (LI-COR 926-68073, 1:5000) secondary antibody. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR) and quantified using Image Studio software (LI-COR). GFP expression was normalized to GAPDH or α-tubulin, which was used as a loading control.

**Luciferase Assay**

Luciferase activity was measured 24 hours after transfection with p5RHH-LUC-mRNA. Cells were rinsed with PBS prior to lysis with Cell Culture Lysis Reagent
(Promega E1531). Luminescence was measured using a Cytation 3 plate reader (BioTEK) using luciferase assay substrate (Promega E1500) per the manufacturer’s protocol.

**miRNA isolation and RT-qPCR**

Total cellular RNA was isolated from cultured cells using miRNeasy mini kit (QIAGEN) according to the manufacturer’s protocol and stored at -80°C in RNase-free water. Reverse transcription was performed on 50ng of total RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and oligo-d(T) or miRNA-specific RT primers (Thermo Fisher Scientific). qPCR was performed using TaqMan MicroRNA Assays (Thermo Fisher Scientific) for 40 cycles on a QuantStudio 3 (Applied Biosystems). Relative expression of miRNAs and mRNA was calculated in comparison to the snoRNA U18 or endogenous GAPDH, respectively, using the 2-∆Ct method.

**Cytotoxicity Assay**

Cytotoxicity was analyzed 24 hours after transfection of miRNA switches by adding propidium iodide (PI) directly to wells without removing the culture media. PI was added to the transfected wells to a final concentration of 4 µM. Fluorescence microscopy images were captured after a 30-minute incubation. The number of dead cells was calculated from 4 fields per well using ImageJ.
**ApoE⁻/⁻ Mouse Model of Atherosclerosis**

Male ApoE⁻/⁻ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in the USF Health Comparative Medicine Vivarium on a 12-hour light/dark cycle with *ad libitum* access to food and water. Animals were placed on a high fat diet (60% kcal from fat) for 18 months prior to use in these experiments. Animals were anesthetized by isoflurane inhalation prior to administration of 200 µl p5RHH-niRFP nanoparticles or vehicle (Optimem) by retro-orbital injection. The animals were sacrificed 48 hours after nanoparticle injection.

**Tissue Distribution Analysis**

ApoE⁻/⁻ mouse organs collected for histological analysis were fixed overnight in 4% PFA followed by an overnight immersion in a cryoprotective 30% sucrose solution as previously described. The organs were then mounted in OCT compound and cryosectioned at 20 µm thickness. Sections were then dried for one hour prior to addition of Prolong Antifade Mountant with DAPI (ThermoFisher). Tissue sections were then examined by fluorescence confocal microscopy for DAPI and niRFP using an Olympus FluoView FV1200. Tissues collected for mRNA analysis were snap frozen after collection and stored in liquid nitrogen until used. Tissues were homogenized and total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was prepared by using oligo-d(t) (NEB) and M-MulV reverse transcriptase (NEB). niRFP transcript level was assayed by SYBR RT-PCR using primers #33 and #34 (Table 4.2). GAPDH, measured using primers #35 and #36 (Table 4.2), was used as a loading control.
Table 4.2 Primers for SYBR RT-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Primer</th>
<th>Forward/Reverse</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td><strong>niRFP</strong></td>
<td>Forward</td>
<td>5’- AAGTCGCTCAGAGAGATGGC</td>
</tr>
<tr>
<td>34</td>
<td><strong>niRFP</strong></td>
<td>Reverse</td>
<td>5’- GAAGCGGTACACACATCACGC</td>
</tr>
<tr>
<td>35</td>
<td><strong>GAPDH</strong></td>
<td>Forward</td>
<td>5’-GTGTTTCTACCCCAATGTGT</td>
</tr>
<tr>
<td>36</td>
<td><strong>GAPDH</strong></td>
<td>Reverse</td>
<td>5’-ATTGTCATACGAGAAATGAGCTT</td>
</tr>
</tbody>
</table>

Male C57BL6/J were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in the USF Health Comparative Medicine Vivarium on a 12-hour light/dark cycle with ad libitium access to food and water. Animals were injected intravenously with 200 µL p5RHH-GFP nanoparticles and sacrificed 48 hours later. Tissues collected for protein expression analysis were snap frozen after collection and stored in liquid nitrogen until analysis by Western blotting.

**Statistics**

Data was analyzed using two-tailed Student’s t-test with Holm-Sidak correction, one-way ANOVA with Tukey’s post-hoc test or two-way ANOVA with Tukey’s post-hoc test using GraphPad Prism 7.0 software. *p* < 0.05 was considered statistically significant. All data are reported as mean ± standard error of the mean (SEM) of at least 3 independent experiments unless stated otherwise.
Chapter 5

Nucleotide modification alters microRNA-dependent silencing of microRNA switches

Note to the Reader

Portions of this chapter have been previously published in Molecular Therapy – Nucleic Acids, 14: 339-350 and have been reproduced here under Creative Commons license CC BY-NC-ND 4.0.

This work was produced in collaboration with Dr. John Canfield, Dr. Ezinne Frances Mong, Jeffrey VanWye, and Dr. Hana Totary-Jain. J.L., J.V., and H.T.-J. designed the research, performed the experiments, and analyzed the data. J.C. and E.F.M. performed experiments and edited the paper. J.L. and H.T.-J. prepared figures and wrote the paper.

Introduction

mRNA therapeutics are becoming valuable tools for the treatment of a broad range of human diseases. Many preclinical and clinical trials are underway using in vitro transcribed (IVT) mRNAs as cancer treatments, vaccines, and protein replacement therapies. mRNA-based therapies represent a remarkable alternative to DNA-based therapies, with a wide range of advantages, including i) increased safety: their non-replicative nature carries no risk for integration into the host genome thus eliminating the chances of genomic alteration; ii) broader applications:
exogenous mRNAs preclude the need for nuclear localization and allows rapid protein expression in any cell type, including non-dividing cells; iii) streamlined production: cell-free systems enable reproducible, rapid, and cost-effective synthesis with stringent quality control. The inclusion of structural elements such as a 5'-cap, 5'- and 3'-UTRs, and a polyA tail has been shown to significantly improve the stability and translational efficiency of IVT mRNA. Additionally, codon engineering and incorporation of chemically modified nucleotides that mimic the naturally occurring intracellular modifications have been shown to reduce immunogenicity and increase translation of IVT mRNA.

To date, 171 types of modified bases have been described, although most types are thought to be rare. Post-transcriptional modifications of RNA are frequent, conserved across species, and observed in different RNA classes. These modifications distinguish endogenous RNA molecules from invading viral or microbial RNA molecules. Pseudouridine (Ψ) is one of the most common nucleotide modification in cellular RNA and is produced by the irreversible isomerization of uridine to Ψ. Hundreds of pseudouridylation sites have been identified in eukaryotic mRNA and have been shown to be dynamically modulated in response to environmental changes and stresses. Ψ affects the stability of endogenous mRNA, indicating potential regulatory roles in mRNA metabolism. Incorporating Ψ into IVT mRNA has been shown to increase translation and decrease immunogenicity. The benefits of incorporating Ψ in IVT mRNA are enhanced when Ψ is combined with other natural modified nucleotides, such as 5-methylcytidine (m5C). Other modifications, such as N1-methylpseudouridine (m1Ψ) have recently been shown to further improve translation
and evasion of the innate immune system. These insights have made IVT mRNA a viable therapeutic option. However, safe and effective mRNA therapeutics require cell-specific targeting to maximize the benefits and minimize off-target effects.

Cell-specific expression has been achieved using various strategies including the incorporation of ligand-sensing aptamers or target sites for endogenous miRNA. The addition of artificial miRNA target sites into the 3'-UTR of the transgene expression vector allows for targeting by the RNA Induced Silencing Complex (RISC) and leads to the silencing of the transgene in cells expressing the corresponding miRNA. We have previously employed this strategy using an adenoviral vector with target sites for miR-126-3p, an endothelial cell specific miRNA, to selectively inhibit the proliferation of arterial vascular smooth muscle cells (VSMC) and prevent restenosis while allowing re-endothelialization of the vessels after balloon injury in a rat carotid artery. Recently, IVT mRNAs that contain artificial miRNA target sites, called miRNA switches, were successfully used to achieve cell-specific expression of synthetic circuits, identify and eliminate undifferentiated induced stem cells, and achieve cell-selective genome editing using miRNA-responsive CRISPR-Cas9.

The intricate mechanisms of miRNA-mediated silencing have been the focus of extensive investigations. However, to date, the relative contributions of mRNA decay and translational repression to the overall silencing remain under debate. Moreover, the influence of chemically modified nucleotides on miRNA-dependent mRNA silencing has not been investigated. Since nucleotide modifications increase translation and stabilize the 2D and 3D RNA structures, thereby altering interactions within the RNA itself and with RNA-binding proteins, we sought to determine whether nucleotide modifications...
affect the regulation of miRNA switches. In this work we compared the effect of nucleotide modifications (Ψ, Ψ/m5C and m1Ψ) on the performance of different miRNA switches in different cell types. We also tested the effect of the location and the complementarity of the miRNA target sites in the miRNA switches.

**Results**

*Nucleotide modifications alter miRNA-dependent silencing of miRNA switches*

To create reporter miRNA switches, we introduced one to four copies of a 22-nt sequence fully complementary to the mature miR-126-3p at the 3’ end of IVT GFP mRNA (GFP-1x126TS, GFP-2x126TS, GFP-3x126TS, GFP-4x126TS). We used Ad-HEK293 cells, since they display minimal cytotoxicity following transfection with unmodified IVT mRNA due to the lack of expression of almost all Toll-like receptors (TLRs)\textsuperscript{102,103,279}. Since Ad-HEK293 cells express very low levels of miR-126-3p compared to human umbilical vein endothelial cells (HUVEC) (Figure 5.1 A), we used exogenous mimics to overexpress miR-126-3p in Ad-HEK293. To determine the optimal time needed for the loading of the miR-126-3p mimics into the RISC, Ad-HEK293 cells were transfected with miR-126-3p mimics simultaneously or 4, 8 or 24 hours prior to transfection with unmodified GFP-4x126TS miRNA switches. miR-126-3p mimics reduced GFP expression to < 37% (\(p < 0.05\)) at all time points compared to vehicle control, and the lowest GFP expression (16.3%) was seen in cells transfected with miR-126-3p mimics 24 hours prior to the GFP-4x126TS miRNA switches (Figure 5.1 B). Therefore, in all subsequent experiments involving Ad-HEK293 cells, we transfected miRNA mimics 24 hours prior to the miRNA switch transfection.
To establish the baseline for miRNA switch silencing, we first assessed GFP expression in Ad-HEK293 cells transfected with unmodified (0% Ψ) GFP mRNA containing zero to four copies of miR-126-3p fully complementary target sites at the 3'UTR in the presence of miR-126-3p mimics, miR-143-3p mimics or vehicle control. In cells transfected with unmodified GFP-1x126TS mRNA, miR-126-3p mimics reduced GFP protein levels to 23.5% ($p < 0.05$) of vehicle control (Figure 5.2 A and B). Increasing the number of miR-126-3p target sites from one to four decreased the expression of GFP protein levels from 23.5% to 6.8% in the presence of miR-126-3p mimics, whereas miR-143-3p mimics showed no difference in GFP expression (Figure 5.2 A and B).

We then synthesized GFP mRNA with 25%, 50%, or 100% substitution of uridine for Ψ. Transfection of these Ψ-modified mRNAs into Ad-HEK293 cells showed >2-fold increase ($p < 0.05$) in GFP expression with 50% and 100% Ψ compared to unmodified (0% Ψ) GFP mRNA (Figure 5.3). We found that increasing Ψ substitutions, from 0% to 100%, reduced miR-126-3p dependent silencing of GFP-1x126TS from 23.5% to 36.4% relative GFP expression (Figure 5.2 A and B). Increasing the number of miR-126-3p target sites from one to four further decreased the expression of GFP protein levels to < 13% at all percentages of Ψ-substitution in the presence of miR-126-3p but not miR-143-3p (Figure 5.2 A and B). The increased silencing of GFP-4x126TS switches compared to GFP-1x126TS was seen at all levels of Ψ substitution, but only reached statistical significance in the 100% Ψ-modified miRNA switches ($p < 0.05$).
Figure 5.1. Expression of miR-126-3p and optimization of miRNA mimic delivery in Ad-HEK293 cells.

(A) Real-time PCR analysis of the indicated miRNA expression in Ad-HEK293 and HUVEC. (B) Representative immunoblot and densitometric quantification of Ad-HEK293 cells transfected with miR-126-3p mimics or vehicle control at the indicated time point before transfection with unmodified GFP-4x126TS miRNA switches. GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM of three independent experiments, normalized against U18 (A) or GAPDH (B) and relative to Ad-HEK293 cells (A) or vehicle control (B). *p < 0.05 versus Ad-HEK293 (A) or 0 hour time point (B). Numbers above bars indicate the mean values.
Figure 5.2. Increasing the number of miRNA-target sites at the 3'UTR of the miRNA switches counteracts the impact of the Ψ-substitution.

(A-D) Representative GFP and GAPDH immunoblots (A and C) and densitometric quantification (B and D) of Ad-HEK293 cells transfected with miR-126-3p, miR-134-3p mimics or vehicle, 24 hours before transfection with the indicated GFP miRNA switch (A and B) or HUVECs transfected with the indicated 100% Ψ-modified GFP miRNA switches (C and D). GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH (B and D) and relative to vehicle controls (B) or untargeted GFP control (D). *p < 0.05 versus miR-143 control (B); versus GFP treated cells (D). #p < 0.05 for the indicated comparisons. “#TS” indicates the number of miR-126-3p target sites in the 3'UTR of the miRNA switch. “NTC” indicates non-transfected control. Numbers above bars indicate the mean values.
Figure 5.3. Ψ-substitution increases GFP expression from IVT mRNA.

(A-B) Representative immunoblot (A) and densitometric quantification (B) of Ad-HEK293 cells transfected with GFP-encoding IVT mRNA with the indicated percentage of Ψ-substitution. GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM of three independent experiments, normalized against GAPDH and relative to unmodified GFP-encoding IVT mRNA. *p < 0.05 versus unmodified GFP-encoding IVT mRNA. Numbers above bars indicate the mean values.
We also assessed the ability of the endogenous miR-126-3p to silence unmodified (0% Ψ) or 100% Ψ-modified GFP miRNA switches containing zero to four copies of miR-126-3p fully complementary target sites at the 3'UTR. HUVECs transfected with unmodified GFP mRNA displayed minimal GFP expression and 3.4-fold (p < 0.05) increase in the number of dead cells compared to non-transfected controls (Figure 5.4 A and B). However, HUVECs transfected with 100% Ψ-modified GFP mRNA displayed increased GFP expression and no difference in the number of dead cells compare to non-transfected controls (Figure 5.4 A and B). Similar to Ad-HEK293 cells transfected with miR-126-3p mimics, HUVECs transfected with 100% Ψ-modified GFP-1x126TS showed 42.3% GFP expression compared to 100% Ψ-modified GFP mRNA (p < 0.05). Increasing the number of miR-126-3p target sites from one to four further decreased the expression of GFP to 25.1% but did not reach statistical significance (Figure 5.2 C and D).

To determine whether the reduction in GFP expression is due to transcript cleavage or reduced translation we analysed GFP transcript levels of HUVECs 24 hours after transfection with 100% Ψ-modified GFP-encoding miRNA switches with zero, one or four fully complementary target sites for miR-126-3p in the 3'UTR. GFP-1x126TS mRNA levels were reduced to 59.8% of cells transfected with 100% Ψ-modified GFP mRNA. Increasing the number of miR-126-3p target sites to four did not further decrease GFP mRNA levels (Figure 5.5), whereas GFP protein levels continued to decrease with the additional target sites (Figure 5.2 C and D). These results show that the additional fully complementary miRNA target sites do not increase the cleavage of
miRNA switches but still contribute to the miRNA-mediated silencing by increasing translational repression.

Lastly, we assessed sponging of the endogenous miRNA by miRNA switches. HUVEC were transfected with 100% Ψ-modified GFP containing zero, one or four miR-126-3p target sites at the 3'UTR, and after 24 hours the expression of miR-126-3p and PIK3R2, a known miR-126-3p target, were determined by RT-PCR. We found that inclusion of one or four miR-126-3p target sites reduced endogenous miR-126-3p levels to ~ 55% (p < 0.05) and increased the mRNA level of PIK3R2 ~1.5-fold (p < 0.05). No difference was observed in the sponging of miR-126-3p or PIK3R2 expression between cells transfected with GFP-1x126TS and GFP-4x126TS (Figure 5.6 A and B). These data indicate that miRNA switches that contain even one fully complementary miRNA target site reduce the availability of the endogenous cognate miRNA and impact the expression of its target genes.

Recent studies have used combinations of modified nucleotides to further decrease the innate immune response to exogenous mRNA\(^{179,274}\). We therefore tested whether 100% substitution with both Ψ and m5C alters miRNA-dependent silencing of GFP miRNA switches that contained zero to four miR-126-3p fully complementary target sites at the 3'UTR. Ad-HEK293 cells transfected with 100% Ψ/m5C GFP-1x126TS showed 18.4% GFP expression (p < 0.05), equivalent to unmodified GFP-1x126TS miRNA switches (23.5%). Similar to unmodified and Ψ-modified miR-126-3p switches, 100% Ψ/m5C-modified GFP-4x126TS produced 14.5% relative GFP expression (p < 0.05) (Figure 5.7 A and B). However, unlike Ψ-modified miRNA
switches, the silencing provided by one miR-126-3p target site in the Ψ/m5C-modified miRNA switches was equal to the silencing of miRNA switches with four target sites.

When the 100% Ψ/m5C-modified GFP mRNA was tested in HUVECs, we observed GFP expression and no difference in the number of dead cells compared to non-transfected controls (Figure 5.4 A and B). HUVECs transfected with 100% Ψ/m5C GFP-1x126TS showed 39.2% GFP expression compared to GFP mRNA controls. As was observed in Ad-HEK293 cells, increasing the number of miR-126-3p target sites at the 3' UTR produced no increase in the silencing (Figure 5.7 C and D). These data demonstrate that in Ψ/m5C-modified miR-126-3p switches, unlike unmodified or Ψ-modified miR-126-3p switches, inclusion of just one miR-126-3p target site at the 3' UTR achieved the same miRNA-dependent silencing as four target sites.

To further explore the influence of the modified nucleotides on the silencing of miRNA switches, we designed GFP mRNA containing four fully complementary target sites at the 3'UTR for miR-21-5p which contains six uridines and five cytosines, (GFP-4x21TS), or miR-145-5p (GFP-4x145TS), which contains four uridines and four cytosines. In contrast miR-126-3p contains six uridines and six cytosines (Figure 5.8 A). Ad-HEK293 cells were transfected with unmodified, 100% Ψ- or 100% Ψ/m5C-modified GFP, GFP-4x21TS or GFP-4x145TS miRNA switches 24 hours after transfection with miR-21-5p, miR-145-5p, miR-143-3p mimics or vehicle control. In cells transfected with unmodified-, Ψ- or Ψ/m5C-modified GFP-4x21TS mRNA switches, only miR-21-5p mimics reduced GFP protein levels to 10.5%, 41.5% and 25.6%, respectively compared to vehicle control (p < 0.05), whereas miR-145-5p or miR-143-3p showed no inhibitory effect (Figure 5.8 B and C).
Figure 5.4. Nucleotide modification decreases cytotoxicity and increases GFP expression in HUVEC.

(A-B) Representative fluorescence microscopy images (A) and number of dead cells per field (B) of HUVEC 24 hours after transfection with GFP-encoding IVT mRNA with the indicated modified nucleotides (Scale bars: 200 μm; 20x magnification). Media was changed four hours after mRNA transfection. Fluorescence Imaging and quantification of dead cells was performed by adding propidium iodide after 24 hours without removal of the media. Data represent the mean ± SEM from four non-overlapping fields. Numbers above bars indicate the mean values.
Figure 5.5. Additional miRNA target sites do not increase cleavage of miRNA switches.

Real Time-PCR analysis of GFP normalized to GAPDH mRNA from HUVEC transfected with the indicated 100% Ψ-modified GFP miRNA switch. Data represent the mean ± SEM of one representative experiment performed in triplicate. *p < 0.05 versus GFP mRNA transfected cells. Numbers above bars indicate the mean values.

Figure 5.6. miRNA sponging by miRNA switches in HUVEC.

(A-B) Real Time-PCR analysis of miR-126-3p normalized to U18 snRNA (A) and PIK3R2 normalized to GAPDH mRNA (B) from HUVEC transfected with the indicated 100% Ψ-modified GFP miRNA switch. Data represent the mean ± SEM of one representative experiment performed in triplicate. *p < 0.05 versus GFP mRNA transfected cells. Numbers above bars indicate the mean values.
Figure 5.7. Ψ/m5C substitution prevents increased silencing by additional miR-126-3p target sites.

(A-D) Representative GFP and GAPDH immunoblots (A and C) and densitometric quantification (B and D) of Ad-HEK293 cells transfected with miR-126-3p, miR-143-3p mimics or vehicle, 24 hours before transfection with the indicated GFP miRNA switch (A and B) or HUVECs transfected with the indicated 100% Ψ/m5C-modified GFP miRNA switches (C and D). GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH and relative to vehicle control. *p < 0.05 versus miR-143 control (B); versus GFP treated cells (D). #p < 0.05 for the indicated comparisons. “#TS” indicates the number of miR-126-3p target sites in the 3'UTR of the miRNA switch. “NTC” indicates non-transfected control. Numbers above bars indicate the mean values.
Similarly, in cells transfected with unmodified-, Ψ- or Ψ/m5C-modified GFP-4x145TS mRNA, only miR-145-5p mimics reduced GFP protein levels to 11.0%, 43.8% and 32.6%, respectively compared to vehicle control (p < 0.05), whereas miR-21-5p or miR-143-3p showed no inhibitory effect (Figure 5.8 B and C). Although the silencing of Ψ- or Ψ/m5C-modified GFP-4x21TS and GFP-4x145TS by miR-21-5p and miR-145-5p mimics, respectively, was less effective compared to unmodified miRNA switches it did not reach statistical significance (Figure 5.8 B and C). We also designed GFP mRNA containing four fully complementary target sites at the 3'UTR for miR-122-5p, which contain 4 uridines and 9 cytosines, and found no difference in GFP silencing of unmodified (9.5%), 100% Ψ- (11.6%) or 100 % Ψ/m5C-modified (20.2%) GFP-4x122TS miRNA switch by miR-122-5p mimics (Figure 5.8 D and E). Taken together these data indicate that, Ψ and Ψ/m5C-modification tend to decrease the silencing of the miRNA switches in comparison to the unmodified miRNA switches.

Regulation of Ψ- and Ψ/m5C-modified miRNA switches is cell type-dependent

We also tested these miRNA switches in cell types that endogenously express the cognate miRNAs to confirm the effects we observed using miRNA mimics in Ad-HEK293 cells. We used HUVEC and VSMC, which express high levels of miR-126-3p and miR-145-5p, respectively in addition to miR-21-5p. We also used HeLa cells, which express high levels of miR-21-5p, but neither miR-126-3p nor miR-145-5p (Figure 5.9 A).
Figure 5.8. Silencing of Ψ- and Ψ/m5C-modified modified miRNA switches is independent of the U or C content.

(A) Sequences of miR-126-3p, miR-21-5p, miR-145-5p, and miR-122-5p and their complementary target sites. ^ denotes the site of Argonaute cleavage. Red bases are complementary to the seed sequence. (B-E) Representative immunoblots (B and D) and densitometric quantification (C and E) of Ad-HEK293 cells transfected with the indicated miRNA mimics or vehicle control 24 hours before transfection with the indicated miRNA switches. GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH and relative to vehicle control. *p < 0.05 versus miR-143 control. “NTC” indicates non-transfected control. Numbers above bars indicate the mean values.
HUVECs transfected with Ψ-modified or Ψ/m5C-modified GFP-4x126TS switches showed a similar reduction in GFP expression, to 42.3% or 30.4% respectively, compared to their respective GFP mRNA transfected controls ($p < 0.05$). However, Ψ-modified GFP-4x21TS miRNA switches reduced GFP expression to 55.2% of Ψ-modified GFP transfected cells ($p < 0.05$), while Ψ/m5C-modified GFP-4x21TS expression was reduced to only 70.5% of Ψ/m5C-modified GFP transfected controls, which did not reach statistical significance. Lastly, HUVECs transfected with Ψ- or Ψ/m5C-modified GFP-4x145TS showed no decrease in GFP expression compared to GFP controls (Figure 5.9 B and C).

In VSMC, transfection of either Ψ- or Ψ/m5C modified GFP-4x126TS switches did not decrease GFP expression compared to Ψ- or Ψ/m5C modified GFP mRNA controls. Ψ-modified GFP-4x21TS switches produced 20.8% GFP expression relative to Ψ-modified GFP mRNA controls ($p < 0.05$), while Ψ/m5C-modification exhibited 73.0% GFP expression relative to the Ψ/m5C-modified GFP mRNA control. Interestingly, Ψ-modified GFP-4x145TS switches exhibited very little silencing (74.2% relative GFP expression, $p > 0.05$) compared to the Ψ-modified GFP mRNA transfected controls and Ψ/5mC-modified GFP-4x145TS switches showed no silencing at all (Figure 5.9 D and E). No statistically significant differences in GFP expression were observed in the Ψ/5mC-modifed GFP-4x21TS and GFP-4x145TS compared to the Ψ-modified switches.
Figure 5.9. Silencing of $\Psi$- and $\Psi$/m5C-modified miRNA switches by endogenous miRNA.

(A) Real-time PCR analysis of the indicated miRNA expression in HUVEC, VSMC and HeLa normalized to U18. (B-G) Representative immunoblots of GFP and GAPDH expression (B, D and F) and densitometric quantification (C, E, and G) of HUVEC (B and C), VSMC (D and E) and HeLa (F and G) cells transfected with the indicated miRNA switch. GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH and relative to GFP transfected control. *$p$ < 0.05 versus GFP transfected cells. Numbers above bars indicate the mean values.
In HeLa cells, Ψ-modified GFP-4x21TS switches produced 50.0% relative GFP expression, while Ψ/5mC-modified GFP-4x21TS switches were further reduced to 39.1% relative GFP expression. No silencing was seen with Ψ- or Ψ/m5C-modified GFP-4x126TS or GFP-4x145TS switches (Figure 5.9 F and G). These data indicate that the silencing of Ψ- or Ψ/m5C-modified miRNA switches may be influenced by the abundance of the cognate miRNA and/or cell type.

Silencing of modified miRNA switches is affected by the degree of complementarity of the miRNA target sites

To determine whether the miRNA dependent silencing of modified mRNA is affected by the degree of complementarity of target sites, we designed a GFP encoding mRNA that included two 7mer-A1 target sites complementary to the seed region of miR-126-3p in the 3'UTR (GFP-2x126seed) and compared it to GFP-2x126TS (Figure 5.10 A). Two target sites were purposely selected to ensure that we could detect any increase or decrease in the ability of miRNA to silence the miRNA switch. In Ad-HEK293 cells transfected with unmodified GFP-2x126seed or GFP-2x126TS, miR-126-3p mimics decreased GFP protein levels to 20.6% or 26.4%, respectively, compared to vehicle controls (Figure 5.10 B and C). Increasing the percentage of Ψ from 0% to 100% reduced the silencing of GFP-2x126seed by miR-126-5p mimics from 20.6% to 44.9% \((p < 0.05)\), in contrast to GFP-2x126TS, which exhibited relatively equal GFP expression at all percentages of Ψ substitution (Figure 5.10 B and C). Cells transfected with Ψ/m5C-modified GFP-2x126seed also exhibited impaired miRNA-dependent silencing compared to unmodified GFP-2x126seed miRNA switches (41.6% vs. 20.6%,
Strikingly, when 100% Ψ- or 100% Ψ/m5C-modified GFP-2x126seed miRNA switches were transfected into HUVEC, we observed no decrease in GFP expression compared to 100% Ψ- or 100% Ψ/m5C-modified GFP mRNA. Whereas, 100% Ψ- and 100% Ψ/m5C-modified GFP-2x126TS reduced GFP expression to 23.9% and 66.3% (p < 0.05), respectively (Figure 5.10 D and E). The silencing of Ψ-modified GFP-2x126TS was significantly stronger than the silencing of Ψ-modified GFP-2x126seed (p < 0.05), but no difference was observed between the Ψ/m5C-modified miRNA switches.

Interestingly, 100% Ψ/m5C-modified GFP-2x126TS was significantly less silenced than 100% Ψ-modified GFP-2x126TS (Figure 5.10 D and E), an effect that was not seen when using GFP-4x126TS miRNA switches in the same cells (Figure 5.9 B and C). These data indicate that nucleotide modifications largely inactivates miRNA switches containing partial complementarity to the mature miRNA sequence.

m1Ψ-modification affects the silencing of miRNA switches

Recently, it has been reported that mRNAs containing m1Ψ-modification outperformed the Ψ- and Ψ/m5C-modified mRNA platforms. Therefore, we compared the silencing of GFP-4x126TS containing 100% m1Ψ substitutions to unmodified, 100% Ψ- or 100% Ψ/m5C-modified miRNA switches in Ad-HEK293 cells. In the presence of miR-126-3p mimics, m1Ψ-modified GFP-4x126TS exhibited 31.8% relative GFP expression (p < 0.05) and was significantly different than unmodified GFP-
4x126TS that showed only 6.8% GFP expression relative to vehicle controls (Figure 5.11 A and B).

In the presence of miR-126-3p mimics, cells transfected with unmodified GFP-4x126TS were only 4% GFP positive at day one, whereas Ψ-, Ψ/m5C- or m1Ψ-modified GFP-4x126TS transfected cells were 22%, 18.7% and 47% GFP positive, respectively. The percent GFP positive cells decreased to < 2% at day five in all miR-126-3p mimic treated groups (Figure 5.12 A-D). These data show that the nucleotide modifications, particularly m1Ψ, increase the translation and extend the duration of protein expression thereby reducing the silencing efficiency of the miRNA switches.

We also tested whether 100% m1Ψ-substitution affects silencing of the other miRNA switches in Ad-HEK293 cells and found that m1Ψ-modified GFP-4x21TS, GFP-4x145TS and GFP-4x122TS, exhibited 19.4%, 32.9%, and 21.0% GFP expression, respectively, in the presence of their cognate miRNA mimic (p <0.05). Unmodified GFP-4x21TS, GFP-4x145TS, and GFP-4x122TS showed 10.5%, 11.0%, and 9.5% relative GFP expression, respectively. Although the m1Ψ-modified miRNA switches tended to be less effectively silenced by the cognate miRNA than the unmodified miRNA switches, these differences did not reach statistical significance (Figure 5.11 C and D).
Figure 5.10. miRNA target site complementary affects the silencing of Ψ- and Ψ/m5C-modified miRNA switches.

(A) Sequences of mature miR-126-3p, fully complementary or 7mer-A1 seed target sites. ^ denotes the site of Argonaute cleavage. Red bases are complementary to the seed sequence. (B-E) Representative immunoblots (B and D) and densitometric quantification (C and E) of Ad-HEK293 cells transfected with miR-126-3p, miR-143-3p mimics or vehicle control 24 hours before transfection with the indicated miRNA switch (B and C) or HUVECs transfected with the indicated miRNA switches (C and D). GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH and relative to vehicle control (C) or GFP transfected cells (E). *p < 0.05 versus miR-143 control (C); versus GFP treated cells (E). # p < 0.05 for the indicated comparisons. Numbers above bars indicate the mean values.
Figure 5.11. Silencing of modified miRNA switches is affected by the location of the miRNA target sites.

(A-F) Representative immunoblots (A, C and E) and densitometric quantification (B, D and F) of Ad-HEK293 cells transfected with the indicated miRNA mimics or vehicle control 24 hours before transfection with the indicated miRNA switches. GFP and GAPDH expression were measured after 24 hours. Data represent the mean ± SEM, normalized against GAPDH and relative to vehicle control. *p < 0.05 versus miR-143 treated control. #p < 0.05 for the indicated comparisons. “NTC” indicates non-transfected control. Numbers above bars indicate the mean values.
Figure 5.12. Long-term kinetics of miRNA switch silencing.

(A-E) Ad-HEK293 cells were transfected with the indicated miRNA mimic or vehicle control 24 hours prior to transfection with GFP-4x126TS miRNA switches with the indicated nucleotide modifications and control Ψ/m5C-modified niRFP mRNA. GFP and RFP expression was analyzed by flow cytometry of 5000 cells every 24 hours after transfection.
Addition of miRNA target sites at the 5'UTR of the miRNA switches counteract the impact of nucleotide modification

A recent report has shown that placing miRNA target sites in the 5'UTR of the miRNA switches produced stronger miRNA-dependent silencing. To test whether the addition of miRNA target sites at the 5'UTR could counteract the impact of nucleotide modifications on the silencing of miRNA switches, we designed GFP-encoding miRNA switches with one fully complementary target site for miR-126-3p in the 5' UTR of human β-globin (5'UTR-1x126TS-GFP). In Ad-HEK293 cells transfected with unmodified 5'UTR-1x126TS-GFP, miR-126-3p reduced GFP expression to 1.1% in the presence of miR-126-3p mimics (Figure 5.11 E and F). The suppression of GFP was equivalent to that of the four miR-126-3p target sites in the 3' UTR (Figure 5.11 A and B). Moreover, cells transfected with 100% Ψ-, 100% Ψ/m5C, and 100% m1Ψ-modified 5'UTR-1x126TS-GFP switches also showed significant silencing by miR-126-3p compared to miR-143-3p controls (9.0%, 10.9%, and 5.7%, respectively p<0.05). These data indicate that placing one fully complementary miRNA target sites at the 5'UTR of miRNA switches eliminates the impact of m1Ψ-modification seen when using four target sites at the 3'UTR.

Discussion

miRNAs have increasingly been used to build regulatory circuits in synthetic biology. miRNA switches have been recently used to enable cell-specific expression of IVT mRNA. Nucleotide modifications are commonly incorporated in these IVT mRNA to decrease immunogenicity and increase translation. In this manuscript we sought to
investigate the influence of modified nucleotides on the performance of miRNA switches. We found that incorporation of \( \Psi \) and \( m1\Psi \), which increase translation, tend to decrease the miRNA-dependent regulation of miRNA switches, while \( \Psi/m5C \) modification enables one miRNA target site at the 3’ UTR to regulate the miRNA switch as effectively as four target sites. We also demonstrated that the effects of \( \Psi \), \( \Psi/m5C \), and \( m1\Psi \) modification are sequence dependent and are not correlated with the proportion of modified nucleotides in the miRNA target site. Furthermore, modified miRNA switches containing seed complementary target sites are poorly regulated by miRNA, while placing the miRNA target site in the 5’ UTR makes the miRNA-dependent silencing largely insensitive to nucleotide modification (Table 5.1).

**Table 5.1. Summary of miRNA switch silencing in Ad-HEK293 cells.**

The mean GFP expression from the indicated miRNA switches relative to the unmodified miRNA switch in the presence of their cognate miRNA mimics.

<table>
<thead>
<tr>
<th></th>
<th>Unmodified</th>
<th>( \Psi )</th>
<th>( \Psi/m5C )</th>
<th>( m1\Psi )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP-1x126TS</strong></td>
<td>0.235</td>
<td>0.364</td>
<td>0.184</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>GFP-2x126TS</strong></td>
<td>0.264</td>
<td>0.35</td>
<td>0.18</td>
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</tr>
<tr>
<td><strong>GFP-3x126TS</strong></td>
<td>0.077</td>
<td>0.236</td>
<td>0.135</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>GFP-4x126TS</strong></td>
<td>0.068</td>
<td>0.123</td>
<td>0.145</td>
<td>0.318</td>
</tr>
<tr>
<td><strong>GFP-4x21TS</strong></td>
<td>0.105</td>
<td>0.415</td>
<td>0.256</td>
<td>0.194</td>
</tr>
<tr>
<td><strong>GFP-4x145TS</strong></td>
<td>0.11</td>
<td>0.438</td>
<td>0.326</td>
<td>0.329</td>
</tr>
<tr>
<td><strong>GFP-4x122TS</strong></td>
<td>0.095</td>
<td>0.116</td>
<td>0.202</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>GFP-2x126seed</strong></td>
<td>0.206</td>
<td>0.449</td>
<td>0.416</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>5’UTR-1x126TS-GFP</strong></td>
<td>0.011</td>
<td>0.09</td>
<td>0.109</td>
<td>0.057</td>
</tr>
</tbody>
</table>

* N/A: Not assessed

The relative contribution of the endonuclease cleavage and/or suppression of translation in the dynamics of miRNA-mediated mRNA silencing has not been fully elucidated, but ultimately, both pathways contribute to the decrease in protein

108
expression. To explore the differences in miRNA-dependent silencing between unmodified and modified miRNA switches we measured changes at the protein level, which also accounts for the increase in mRNA translation seen with the modifications. We explored different methods for measuring protein expression (flow cytometry and immunoblotting) and carefully quantified and pooled the data when possible. Although immunoblotting is not the most sensitive method of measuring protein expression but it was sufficient for our initial assessments. Further studies that use more sensitive assays are necessary to pinpoint the exact mechanisms by which nucleotide modification alters miRNA-dependent silencing. We also took advantage of the fact that Ad-HEK293 cells lack expression of most TLRs \(^{102,103,279}\) and tolerate transfection with unmodified IVT mRNA. Although Ad-HEK293 cells displayed minimal toxicity when transfected with unmodified IVT mRNA, we noticed lower expression of Ψ/m5C-modified niRFP mRNA when co-transfected with unmodified GFP-4x126TS (Figure 5.12 A). This trans-repression is likely due to the presence of other pattern recognition receptors such as Protein Kinase R (PKR), which phosphorylates the eukaryotic translation initiation factor eIF2a and leads to translation inhibition \(^{280}\). Previous studies have performed similar experiments in Ad-HEK293 cells to show that incorporation of modified nucleotides in IVT mRNA reduces PKR activation, 2'-5'-oligoadenylate synthetase and RNase L activity \(^{102,105,108}\).

Here we found that 100% Ψ substitution doubled the expression of IVT-mRNA compared to unmodified mRNA and slightly reduced miRNA-mediated silencing of all the tested miRNA switches but did not reach statistical significance. Similar results were also observed with m1Ψ-modification, which is known to produce even more protein
expression than Ψ-modification, and it significantly reduced the miRNA-dependent silencing of miR-126-3p switches. These data are in line with previous reports showing that incorporation of Ψ or m1Ψ in IVT mRNA enhanced translational efficiency.103,106,281 Moreover, alterations in mRNA translation initiation, for example tethering of the translation factors eIF-4E or eIF-4G to an mRNA, have been shown to confer resistance to miRNA-induced repression.282 We also found that the increased silencing of Ψ-modified miRNA switches with multiple fully complementary target sites at the 3’ UTR was due to an increase in translational repression rather than transcript degradation. Moreover, miRNA switches drastically reduced the availability of the endogenous miRNA. The miRNA sponging was similar when using miRNA switches with one or four fully complementary target sites. Previous studies using plasmids or viral vectors have reported no sponging activity even when using four fully complementary target sites.276 The differences in miRNA sponging might be due to the temporal expression between the two delivery systems. Plasmids and viral vectors are gradually transcribed and transported to the cytoplasm are less likely to sponge the miRNA, whereas transfection of miRNA switches can immediately bind and potentially saturate the available miRNA due to rapid delivery of a high number of transcripts into the cytoplasm. However, because miRNA switches are transient, the sponging caused by the miRNA switches is temporary.

Similar to Ψ-modified mRNA, Ψ/m5C-modified mRNAs are also known to enhance protein expression and reduce immunogenicity.103 However, in contrast to Ψ-modified miRNA switches, the addition of one miRNA target site to the 3’UTR produced miRNA-dependent silencing of Ψ/m5C-modified miR-126-3p switches as effectively as
when four target sites were used. Moreover, this effect was detected not only in Ad-HEK293 cells, but also occurred in HUVECs. The proportion of m5C in the target sites of the tested miRNA switches did not correlate with the miRNA-dependent silencing efficiency, and the silencing efficiency also varied in different cell types used in this work. Nucleotide modification alters the secondary or tertiary structure of the mRNA which may alter accessibility of the miRNA target sites in a switch-specific manner. In fact, Ψ-modification has been reported to reduce protein binding to consensus sequences in mRNA. The nucleotide modifications may also affect the kinetics of miRNA switch shuttling in or out of p-bodies. Further studies are necessary to explore the interaction of miRNA target site sequence and the changes in miRNA-dependent silencing caused by nucleotide modifications, especially because previous reports have shown enrichment of m5C at site of Argonaute binding and differences in protein expression from modified mRNA in different cell types.

The design of miRNA target sites and its location in a miRNA switch can have a large impact on the capacity for miRNA-mediated silencing. Our results showed that nucleotide modifications have a larger effect on the silencing of miRNA switches that utilize target sites complementary to the seed sequence of the mature miRNA. Because the majority of miRNA target sites in animals utilize seed complementary target sites, post-transcriptional modification of mRNAs may provide an additional level of control over gene expression by tuning miRNA activity. An important feature of the miRNA:mRNA interaction is the thermal stable base-pairing between the miRNA 5’ end (residues 2-7) and the mRNA target. In addition, efficient endonuclease cleavage by Argonaute 2 requires base pairing at the site of cleavage, between bases 10 and 11.
Previous reports have shown that miRNA-mediated silencing primarily occurs though direct cleavage of the targeted mRNA by Argonaute 2 when bound to a fully complementary target site. However, the silencing of partial complementary target genes occurs by translational repression and/or mRNA decay in a manner independent of endonucleolytic cleavage. Binding of the RISC has been show to recruit several proteins including GW182, which can mediate poly(A)-binding protein displacement, recruit translational repressors and/or dissociate eIF4A from the cap-binding complex eIF4F.

Additionally, we show that placing one fully complementary miRNA target site at the 5' UTR eliminates the influence of the nucleotide modifications seen with the four target sites at the 3'UTR of the miRNA switches. The increase in silencing efficiency when using miRNA target sites at the 5' UTR was most pronounced in the m1Ψ-modified miR-126-3p switches. These data are in agreement with previous work that showed that modified miRNA switches with four miRNA target sites at the 3'UTR are less effective than one target site at the 5'UTR. Silencing of miRNA switches by miRNA target sites in the 3'UTR and the 5'UTR has been shown to involve deadenylation and cap-dependent translation inhibition. However, the enhanced silencing of the modified miRNA switches with one fully complementary miRNA target site at the 5'UTR indicates that there may be another mechanism triggered by miRNA target sites in the 5'UTR. One potential explanation is that direct Argonaute 2-mediated cleavage of the targeted miRNA switch upstream of the start codon may be more effective in silencing due to the removal of initiation factors that are bound to the 5' cap; whereas cleavage at the 3' UTR promotes transcript degradation by removing the
stabilizing poly(a)-tail. It is also possible that RISC binding at the 5′ UTR interferes with translation machinery through steric hindrance of ribosome assembly. Furthermore, a miRNA target site near the start codon may be more accessible to the RISC as it has less secondary structure than other regions of mRNA. Further studies are needed to fully explain the enhanced activity of miRNA target sites at the 5′ UTR.

Based on the data presented here, several important considerations must be taken into account when designing effective modified miRNA switches. First, it is crucial to ensure that the selected miRNA is expressed in a cell-specific manner. Second, the endogenous expression levels of the selected miRNA must be abundant. Third, use a fully complementary miRNA target sites and not miRNA seed sequences. Fourth, including multiple miRNA target sites increases the miRNA-mediated suppression of the miRNA switch. Fifth, placing the miRNA target sites at the 5′UTR will result in effective miRNA-dependent silencing and eliminate the reduction in silencing caused by nucleotide modification. However, switches with miRNA target sites at the 5′UTR increase translation in some instances and therefore should be tested to ensure switch activity. Lastly, the dose of miRNA switches should be fine-tuned to prevent saturation of endogenous miRNA.

The present study is of broad significance given the dynamic changes in the naturally occurring modified nucleotides in all cellular mammalian RNA. The impact of these modifications on miRNA-mediated silencing provides a new mechanism of gene regulation at the epitranscriptome level, which has not been explored. This work provides insights into the influence of the naturally occurring nucleotide modifications on miRNA-dependent silencing and informs the design of optimal miRNA switches.
Furthermore, given that modified nucleotides increase translation, they can serve as a novel tool to elucidate the relative contribution of the endonuclease cleavage and/or suppression of translation in the dynamics of miRNA-mediated mRNA silencing.
Chapter 6

Systemic administration of self-assembled p5RHH-based nanoparticle delivers synthetic mRNA to plaques in a mouse model of atherosclerosis

Note to the Reader

This work was produced in collaboration with Jeffrey VanWye, Dr. John Canfield, Dr. Ezinne Fransess Mong, Dr. Samuel Wickeline, Dr. Hua Pan, and Dr. Hana Totary-Jain. H.T.J. conceived the idea. J.L., J.V. and H.T.J. designed the research, performed the experiments, and analyzed the data. J.C. and E.M. performed the experiments. J.L. and H.T.J. wrote the paper. S.W. and H.P. provided conceptual and practical advice on experimental plans, contributed reagents and mice, and reviewed all data. All authors reviewed and edited the manuscript.

Introduction

RNA therapeutics are a promising new class of biological drugs that are under active development for a variety of human diseases. mRNA therapeutics in particular have a variety of potential applications, including protein replacement therapy, CRISPR/Cas9 gene editing, and vaccines against various viruses. The use of mRNA therapeutics offers advantages over traditional DNA or viral gene therapy vectors as there is no risk of insertional mutagenesis, and the rapid expression of the encoded proteins does not require cellular division. In addition, mRNA therapeutics can encode any gene regardless of the length of the coding sequence to yield proteins with
appropriate post-translational modifications. For example, the cell-free production and the tight quality control possible with mRNA therapeutics has proven beneficial in the rapid generation of mRNA-based vaccines in response to disease outbreaks 171,302.

Despite recent advances in RNA structural chemistry 86,303, the safe and efficient delivery of the RNA molecules remains a major hurdle. RNA molecules are highly charged and too large to cross cellular membranes on their own. While synthetic RNA molecules may be taken up by cells via endocytosis, they produce little biological activity due to endosomal entrapment and degradation in lysosomes. Modifications to the phosphodiester backbone may enhance endosomal escape but only for small single-stranded RNA such as siRNA 304. Larger RNA molecules such as mRNA can be delivered to cytoplasmic compartments by physical transfection methods such as microinjection or electroporation, but these approaches are unsuitable for systemic delivery in vivo.

Cationic lipids and polymers can package RNA into nanoparticles that are taken up by cells to effect a measure of endosomal escape. However, the strong positive charge carried by these nanoparticles promotes opsonization by serum proteins and uptake by the mononuclear phagocyte system, particularly by Kupffer cells in the liver 305. In recent years, extensive work has been dedicated to the refinement of these nanoparticles by using ionizable lipids and polymers that are only cationic in acidic environments such as maturing endosomes 306. While pH sensitive nanoparticles exhibit reduced liver toxicity compared to cationic nanoparticles, they still become trapped in the liver. Modulating the composition of the nanoparticles by including different lipids or adding targeting components to the nanoparticle shell can provide a degree of organ
specificity. However, synthesis of such nanoparticle systems can be complex, requiring precise microfluidic assembly followed by separation and purification steps.

We recently designed and tested a modified and systemically safe version of the natural cell penetrating peptide melittin by amino-terminus truncation and inclusion of positively charged residues at the C-terminus, named “p5RHH”, that rapidly interacts with short negatively charged oligonucleotides such as siRNA to form 55 nm particles spontaneously upon mixing. These nanoparticles are taken up by macropinocytosis and traffick through endosomes. As the interior pH of endosomes drops due to vacuolar ATPase (vATPase) activity during maturation, the histidine residues become protonated and the p5RHH nanoparticles disassemble, perhaps due to the repulsive force of the greater cationic charge overcoming the hydrophobic force that gives the nanoparticle its structure. The free p5RHH, which is membrane-lytic only in high concentrations in the endosome, induces endosomal lysis and release of the siRNA. Importantly, the concentration of the free p5RHH released in the cytoplasm is insufficient to disturb the plasma membrane, which maintains cell viability.

The p5RHH-siRNA nanoparticles are effective in targeting NFκB subunit p65 in mouse models of arthritis, JNK2 in the plaques of atherosclerotic mice and AXL in a xenograft mouse model of ovarian cancer. However, the ability of p5RHH to bind mRNA, form nanoparticles and effectively deliver it to the cellular cytoplasm to be translated has not been tested. The present study demonstrates that p5RHH spontaneously forms nanoparticles in the presence of synthetic mRNA. These nanoparticles were able to protect the mRNA from degradation and efficiently deliver it to cells to be translated. Moreover, systemic administration of p5RHH-near infrared
florescent protein (niRFP) encoding mRNA nanoparticles in ApoE knockout mice resulted in high expression of niRFP only in the atherosclerotic plaque regions, whereas niRFP was not detected in regions of intact endothelium or other organs.

Results

p5RHH spontaneously forms nanoparticles with synthetic mRNA

The p5RHH comprises 21 amino acids with a hydrophobic core and a cationic C-terminus tail that contains 5 arginine and 2 histidine residues (Figure 6.1 A). The C-terminus of p5RHH carries a strong positive charge that enables the peptide to interact electrostatically with the negatively charged backbone of nucleic acids. To examine the interaction of p5RHH with mRNA, a gel retardation assay was performed using 350 ng in vitro transcribed Cy5-uridine labeled GFP-coding mRNA (to allow for visualization) incubated with increasing amounts (0 - 2.5 nmol) of p5RHH. Given the positive charge of p5RHH, we anticipated that only free unbound mRNA would migrate when an electric field was applied. As expected, a single band of ~1100 nt migrated in the agarose gel when free Cy5-uridine labeled GFP mRNA was loaded. Incubation of Cy5-labeled GFP mRNA with increasing amounts of p5RHH decreased the free mRNA signal and shifted the Cy5 signal to the loading well, indicating that the Cy5-labeled GFP mRNA was bound to the p5RHH peptide and formed a complex that did not migrate through the agarose gel when the electrical field was applied (Figure 6.1 B).

To determine the optimal conditions for the formation of nanoparticles, 350 ng GFP mRNA was mixed with 2 nmol p5RHH and incubated at 4, 20 or 37°C for 40 minutes, or incubated at 37°C for 5, 10, 20, 40, 60 or 120 minutes before adding it to
B16F10 cells. After 24 hours, the percentage of GFP positive cells was determined by flow cytometry. Incubation temperature of 37°C and incubation times of 20 or 40 min displayed the highest transfection efficiencies (Figure 6.1 C,D). Therefore, all nanoparticles in the subsequent experiments were tested under these conditions.

We also tested the duration of GFP expression over time in B16F10 cells transfected with GFP-p5RHH and detected GFP positive cells by flow cytometry up to nine days after transfection (Figure 6.1 E).

mRNA and p5RHH form consistent nanoparticle size regardless of mRNA length

To determine the optimal mRNA-p5RHH ratios that results in the highest transfection efficiency and the lowest cytotoxicity, 714 nt in vitro transcribed GFP mRNA (350 ng) was incubated with increasing amounts (0 - 2.5 nmol) of p5RHH, and B16F10 cells were transfected with the resulting nanoparticles. After 24 hours, GFP- and propidium iodide- (PI) positive cells were determined by flow cytometry. Cells transfected with GFP mRNA complexed with increasing amount of p5RHH resulted in an increased percentage of GFP positive cells that reached a maximum of 75% at 2 nmol p5RHH (Figure 6.2 A).

We also tested whether the optimal mRNA-p5RHH ratios is affected by mRNA length. Therefore, 350 ng of mRNA encoding near infrared fluorescent protein 670 (niRFP, 936 nt), firefly luciferase (Luc, 1653 nt), or self-replicating mRNA derived from the Venezuelan equine encephalitis virus expressing GFP (VEE-GFP, 8600 nt) were incubated with increasing amounts (0 - 2.5 nmol) of p5RHH. After 24 hr, transfection efficiency of niRFP mRNA-p5RHH and VEE-GFP mRNA-p5RHH, as well as luciferase
activity from Luc mRNA-p5RHH all peaked at the same ratio of 350 ng mRNA to 2.0 nmol p5RHH, similar to the 714 nt GFP mRNA-p5RHH (Figure 6.2 A-D). However, the transfection efficiency of VEE-GFP mRNA-p5RHH was lower than the other nanoparticles (Figure 6.2 D). Therefore, in all subsequent experiments we used a ratio of 350 ng synthetic mRNA to 2 nmol p5RHH.

To visualize and measure the diameter of each of the different mRNA-p5RHH nanoparticles, scanning electron microscopy imaging was performed at the optimized ratio of mRNA to p5RHH (350 ng mRNA : 2.0 nmol p5RHH). Strikingly, compact spherical nanoparticles with an average diameter of < 200 nm were observed for each of the tested mRNA (Figure 6.2 E-H). Interestingly, the niRFP, Luc, and VEE-GFP mRNA nanoparticles were very similar sizes, while the GFP mRNA nanoparticles were significantly smaller (Figure 6.2 E-H). Despite the difference in sizes, the zeta potential measurement of the different mRNA-p5RHH nanoparticles showed an effective surface charge of approximately +6 mV for each nanoparticle. These results demonstrate that the physical characteristics of mRNA-p5RHH nanoparticles are consistent even when formed with different mRNA payloads, which may increase the reproducibility of nanoparticle transfections in vitro and in vivo.
Figure 6.1. p5RHH spontaneously forms nanoparticles when mixed with synthetic mRNA.

(a) Predicted structure, hydrophobicity and charge density plots for p5RHH. (b) mRNA loading assay of 350ng of 5% Cy5-uridine labeled GFP mRNA (~1000nt) complexed with the indicated amounts of p5RHH. Cy5 signal is shown in red, ethidium bromide staining is shown in white. (c-d) Flow cytometry of B16F10 cells 24 hours after transfection with GFP mRNA-p5RHH nanoparticles complexed for 40 minutes at the indicated temperature (c) or the indicated time at 37 °C (d). (e) Flow cytometry of B16F10 cells at the indicated number of days after transfection with p5RHH-GFP nanoparticles. Cells were re-plated at a 1:5 dilution on day 5. Data represent the mean ± SEM of three independent experiments.
Figure 6.2. p5RHH-mRNA nanoparticles with payloads of various lengths.

(a-d) Flow cytometry (a, b and d) and luciferase assay (c) of B16F10 cells 24 hours after treatment with the indicated amount of p5RHH complexed with 350 ng of GFP (a), niRFP (b), Luc (c) or VEE-GFP (d) mRNA. (e-h) Representative scanning electron microscopy images accompanied by dot plot of sizes of p5RHH nanoparticles loaded with GFP (e), niRFP (f), Luc (g) or VEE mRNA (h) on a polycarbonate membrane with 200 nm pores (dark circles). Data represent the mean ± SEM of three independent experiments (a-e).
Effective mRNA release of the mRNA-p5RHH nanoparticles require endosomal acidification

To confirm that mRNA-p5RHH nanoparticles traffic through endosomes, B16F10 cells were transfected with 5% Cy5-labeled GFP mRNA-p5RHH nanoparticles. Cells were treated with 1 µM bafilomycin, an inhibitor of endosomal acidification, or vehicle control. Particle uptake, measured by percent Cy5-positive cells, and percent GFP-positive cells were determined by flow cytometry at 40 minutes and 24 hours after transfection. While no GFP-positive cells were detected after 40 min of transfection, ~65% of the cells were Cy5-positive in both bafilomycin treated or untreated cells, indicating that bafilomycin does not affect cellular uptake of the nanoparticles (Figure 6.3 A,B). After 24 hours, 56.7% of the untreated cells were GFP-positive, whereas bafilomycin treated cells exhibited only 14.6% (p< 0.05), even though the percent of Cy5-positive cells was still at ~65% in both groups (Figure 6.3 A,B). These data indicate that mRNA-p5RHH nanoparticles are rapidly taken up by cells, traffic through the endosomes and require endosomal acidification to efficiently transfect cells.

mRNA-p5RHH nanoparticles possess inherent endosomal escape

Next, we tested whether addition of chloroquine, a known endosomolytic agent, enhanced the transfection efficiency by inducing endosomal release of the mRNA-p5RHH nanoparticles that may be still trapped in the endosomes. Again, B16F10 cells were transfected with 5% Cy5-uridine labeled GFP mRNA-p5RHH nanoparticle and treated with vehicle or with 50 µM chloroquine. The percentages of Cy5- and GFP-positive cells were determined by flow cytometry 40 minutes and 24 hours after
transfection. After 40 min of transfection, cells treated with chloroquine showed no significant difference in the percent of Cy5-positive cells compared to vehicle treated cells (67.2% vs. 65.1%) and no GFP-positive cells were detected, indicating that chloroquine does not affect cellular uptake of the nanoparticles (Figure 6.3 C,D). Importantly, after 24 hours, despite the small increase (76.9% vs. 69.3%) in Cy5-positive cells, no change in the percent of GFP-positive cells (56.8% vs. 55.7%) was found between chloroquine treated or untreated cells (Figure 6.3 C,D) indicating the efficient transfection efficiency of GFP mRNA-p5RHH nanoparticles is due to the intrinsic membrane-lytic activity of p5RHH 241.

**mRNA-p5RHH nanoparticles does not induce cytotoxicity**

To ensure that the endosomolytic activity of the mRNA-p5RHH nanoparticles does not induce apoptosis of the transfected cells, we measured the percent of propidium iodide (PI)- positive B16F10 cells after transfection with GFP-mRNA p5RHH nanoparticles at various mRNA to peptide ratios. After 24hr we observed no significant increase in PI-positive cells at any of the tested ratios (Figure 6.4 A,B), indicating that the unbound p5RHH released after endosomolysis does not induce significant cell death.
Figure 6.3. Endosomal escape of p5RHH-mRNA nanoparticles.

(a-d) Flow cytometry of B16F10 cells at the indicated timepoint after treatment with 5% Cy5-uridine labeled GFP mRNA-p5RHH nanoparticles in the presence 1 µM bafilomycin (a and b) or 50 µM chloroquine (c and d). Data represent the mean ± SEM of three independent experiments. * p < 0.05 nanoparticle + vehicle treated cells (b and d).
Figure 6.4. mRNA-p5RHH nanoparticles exhibit minimal cytotoxicity and RNase sensitivity.

(a and b) Flow cytometry with propidium iodide of B16F10 cells 24 hours after treatment with mRNA-p5RHH nanoparticles formed by complexing 350 ng of GFP mRNA with the indicated amount of p5RHH. (c) Gel electrophoresis of unbound and p5RHH-complexed Cy5-labeled GFP mRNA with or without RNaseA treatment after nanoparticle formation. (d) Flow cytometry of B16F10 cells 24 hours after treatment with GFP mRNA-p5RHH nanoparticles with or without RNaseA treatment after nanoparticle formation. Pretreatment of the GFP mRNA prior to nanoparticle formation was used as a control for RNaseA activity. Data represent the mean ± SEM of three independent experiments. * p < 0.05 versus untransfected control (b) or no RNase treatment (d).
mRNA-p5RHH nanoparticles protect the mRNA from degradation by RNase

Successful in vivo delivery of mRNA therapeutics requires stable nanoparticles that can protect the synthetic mRNA from degradation. To test whether mRNA-p5RHH nanoparticles protect their payload from degradation by RNaseA, we treated Cy5-labeled GFP mRNA-p5RHH or free Cy5-labeled GFP mRNA with vehicle control or with RNaseA for 20 min in 37°C before analyzing the resulting products by gel electrophoresis. Free mRNA exhibited extensive degradation by RNaseA, whereas the mRNA complexed with p5RHH showed no mRNA degradation (Figure 6.4 C). Treatment of GFP mRNA-p5RHH nanoparticles prior to adding them to the B16F10 cells showed no difference in percent GFP positive cells between the RNase treated or untreated nanoparticles after 24hr (Figure 6.4 D). However, pretreatment of the GFP mRNA with RNaseA before formation of the nanoparticles abolished GFP expression (Figure 6.3 D). These results indicate that mRNA-p5RHH nanoparticles may withstand serum endonucleases, which is critical for in vivo delivery of mRNA therapeutics.

mRNA-p5RHH nanoparticles exclusively target atherosclerotic plaques in ApoE-/- mice

To test the efficacy of mRNA-p5RHH nanoparticles in vivo, C57BL6/J mice were injected intravenously with GFP mRNA-p5RHH nanoparticles and GFP expression was assessed after 48 hours by immunoblotting lysates from the brain, heart, lungs, muscle, pancreas, liver, spleen, kidneys and bladder. No GFP expression was detected in any of these organs (Figure 6.5). Consistent with these results, previous studies have showed that siRNA-p5RHH nanoparticles localize only to regions of disrupted endothelial cell barriers in advanced atherosclerotic plaques after systemic
administration. Therefore, we tested whether mRNA-p5RHH nanoparticles could deliver a reporter mRNA in an ApoE-/ mouse model of atherosclerosis. To avoid the autofluorescence that might mask GFP signal, mice received intravenous injection of vehicle control or niRFP mRNA-p5RHH nanoparticles. After 48 hours, niRFP expression in the different regions of the aorta were examined by en face confocal imaging. To assess endothelial cell integrity, aortas were immunostained for the endothelial cell specific marker VE-Cadherin. Strong niRFP expression was found in advanced atherosclerotic plaques located at the aortic arch regions of the treated mice, whereas thoracic regions that did not develop plaques exhibited no niRFP expression (Figure 6.6). Importantly, the kidneys, liver, lungs and spleen collected from the niRFP mRNA-p5RHH nanoparticle treated ApoE-/- mice showed no niRFP signal by confocal microscopy (Figure 6.7). In addition, we no niRFP mRNA could be detected in any of these organs by RT-PCR. These results highlight the potential for treating atherosclerosis with mRNA therapeutics utilizing the specificity and efficiency of mRNA-p5RHH nanoparticles.
Figure 6.5. Tissue distribution of p5RHH-GFP nanoparticles in C57BL6/J mice.

Representative immunoblots of GAPDH and GFP in protein lysates of the indicated organs collected from three C57BL6/J mice 48 hours after intravenous injection of 200 µL of GFP mRNA-p5RHH nanoparticles. B16F10 cells transfected with the same complexing reaction for each animal were used as a GFP+ control. An untreated mouse was used as control for antibody specificity.
**Figure 6.6. Expression of p5RHH-mRNA nanoparticle payloads in the aorta of ApoE\(^{-}\) mice.**

Representative confocal images of aortic arch plaques and regions of the thoracic aorta captured from *en face* preparations of ApoE\(^{-}\) aortas 48 hours after vehicle or niRFP mRNA-p5RHH nanoparticle injection. Images were captured at 20x magnification, insets magnified to 80x. Scale bars represents 100 µm. White arrowheads indicate niRFP expressing cells.
Figure 6.7. Tissue distribution of p5RHH-niRFP nanoparticles in ApoE−/− mice.

Representative fluorescent confocal microscopy images of the indicated organs collected from ApoE−/− mice 48 hours after intravenous injection of 200 µL of vehicle or niRFP mRNA-p5RHH nanoparticles. Images were taken at 20x. Scale bar represents 100 µm.
Discussion

Synthetic mRNA therapeutics hold great promise for the treatment of myriad diseases. Despite substantial advances in the production of the synthetic mRNA, the limitations of existing delivery platforms severely hinder its clinical development. Identification of novel delivery vehicles that prevent degradation and effectively deliver the mRNA are crucial for the expansion of this promising class of therapies. The present study demonstrates that p5RHH peptides in the presence of mRNA spontaneously form compact nanoparticles across a range of mRNA sizes. These nanoparticles are highly RNase resistant, readily taken up by the cells, and efficiently and safely release the mRNA from the endosomes to be translated. Moreover, systemic delivery of p5RHH-niRFP nanoparticle in atherosclerotic mice generated high expression levels of the synthetic mRNA exclusively in atherosclerotic plaque regions.

The natural cell-penetrating peptide melittin is the major pore-forming component of bee venom. In prior work, we substantially modified its cell lytic activity by amino-terminus truncations to produce a new peptide, “p5”, which still allowed it to stably but safely insert into cell membranes. This p5 was developed as a linker agent for delivery of various therapeutic or diagnostic compounds into cells by the penetrating action of the peptide. However, neither p5 nor melittin itself were able to condense siRNA into a nanoparticle that was transfective (unpublished data). Upon further highly specific modification of the C-terminus with histidines and arginines to create p5RHH, a transfective particle was enabled for siRNA delivery.

Alternative formulations of the native membrane lytic melittin have been reported to promote endosomal escape and efficient release of the DNA into the cytoplasm after
covalent attachment to polyethylenimine. In this case, to limit the inherent toxicity of native melittin to endosomes, additional pH-sensitive protecting groups were conjugated to mask melittin’s pore forming activity at neutral pH, which then could be removed upon endosomal acidification. However, this strategy ultimately proved to exert unacceptable levels of toxicity, prompting the termination of clinical trials against Hepatitis B (NCT02452528).

In contrast, the p5RHH is an N-terminal truncation of melittin that retains a 13 aa hydrophobic core combined with a modified cationic C-terminus composed of five arginine and two histidine residues. The p5RHH peptide initiates formation of nanoparticles through electrostatic interactions with negatively charged siRNA molecules, which remain stable in circulation as a complex at neutral pH. As the peptide is not free, its cytotoxicity is minimized until the entire particle is disassembled in the endosome at lower pH. In analogous fashion, the p5RHH rapidly interacts with mRNA molecules of different sizes to self-assemble nanoparticles with high transfection efficiency and minimal cytotoxicity. Despite the 50-fold difference in GFP mRNA to siRNA length or even 400-fold difference in the case of the VEE-GFP mRNA, the optimal charge ratio of p5RHH and synthetic mRNA (+10:-1) was similar to that previously reported for p5RHH and siRNA (+12:-1), because the mass-to-charge ratio of nucleic acids is essentially constant. Therefore, the ratio of 350 ng synthetic mRNA to 2 nmol p5RHH, which produces the optimal charge ratio, might be scaled up or down to deliver a range of lengths of RNA, from siRNA to 8kb long mRNA, and perhaps beyond.
Measurement of the p5RHH-mRNA nanoparticles size and surface charge revealed compact spherical structures that were <200 nm in diameter with a +6 mV zeta potential for all of the tested mRNA. Because the cell membrane is a negatively charged surface, the nanoparticles’ positive surface charge may increase cellular uptake by endocytosis\textsuperscript{307}. In addition, the small magnitude of the surface charge may minimize opsonization by serum proteins and clearance by the mononuclear phagocyte system, thereby avoiding sequestration in the liver and spleen. Prior work with p5RHH-siRNA nanoparticles has shown that uptake is mediated by macropinocytosis and trafficks through endosomes\textsuperscript{241}. Given the similar formation kinetics and in vivo behaviors of the p5RHH-mRNA nanoparticles, we propose that the same mechanisms for cellular interactions would be responsible for p5RHH-mRNA nanoparticles.

 Trafficking of synthetic mRNAs from endosomes into the cytoplasm represents a major rate-limiting step for many delivery approaches, with typical endosomal escape efficiencies of around 1-2\%\textsuperscript{218}. The present study demonstrates that the endosomal escape of p5RHH-mRNA nanoparticles is highly efficient since inducing complete endosome lysis with chloroquine did not potentiate transfection. We also show that the intrinsic endosomolytic activity of p5RHH-mRNA nanoparticles requires endosomal acidification to release the payload mRNA into the cytosol, where it can be rapidly translated into protein. Indeed, we observed that the entire process from addition of the p5RHH-mRNA nanoparticles to visible GFP expression takes less than one hour in vitro.

 Previous work by Hou et al has shown that protonation of the two histidine residues (pKa \~ 6) of p5RHH is necessary for the dissociation of the p5RHH peptide
from the nucleotide payload $^{240,241}$. The pH-dependent dissociation of p5RHH-siRNA nanoparticle was confirmed in vitro by using a dye-binding assay which showed increasing accessibility to the free siRNA at pH ≤ 5.5 that promoted particle disassembly $^{241}$. Once the p5RHH is dissociated from the siRNA upon endosomal acidification, the free p5RHH causes endosomal disruption, which was confirmed by release of the endosomal dye acridine orange into the cytoplasm of p5RHH-siRNA nanoparticle treated cells. Importantly, a p5RHH analog peptide p5RWR, that lacks the ionizable histidine residues was able to form nanoparticles with siRNA but failed to dissociate from its siRNA payload and did not alter endosomal integrity. Therefore, the pH-sensitive histidine residues are both necessary and sufficient for disassembling p5RHH-mRNA nanoparticles, while enabling release of the peptide to permeabilize endosomes after acidification.

Regarding mechanism, the buffering capacity of p5RHH conferred by the ionizable histidine residues is far lower than necessary to lyse the endosomes through osmotic pressure or “proton sponging”. Instead, p5RHH directly interacts with the endosomal membrane leading to endosomolysis via membrane destabilization $^{234}$. Because p5RHH was engineered deliberately to be far less potent as a membrane disruptor in comparison to native melittin as shown earlier by Pan et al it only effects membrane permeability in the high concentrations found in the endosome after particle disassembly $^{239,308,309}$.

The present study also demonstrated that p5RHH-mRNA nanoparticles exhibit minimal cytotoxicity. The binding of p5RHH to the mRNA reduces the concentration of the free p5RHH prior to dissociation in the endosome. After endosomolysis, the
concentration of the free p5RHH is diluted in the cytosol, preventing disruption of the cell membrane. Previous work with pH-responsive native melittin adjuvants by Arrowhead Pharmaceuticals has demonstrated an unacceptable level of hepatotoxicity after systemic administration, leading to halting of a Phase 2 clinical trial for Hepatitis B (NCT02452528), although trials have resumed using this system for subcutaneously delivery (NCT03747224, NCT03365947). However, p5RHH has not been reported to produce any such toxicity due to the designed attenuation of its membrane lytic capacity and the avoidance of liver sequestration of the p5RHH-siRNA and p5RHH-mRNA nanoparticles.

Another key requirement for an effective in vivo mRNA delivery platform is the ability to prevent degradation of the synthetic mRNA by extracellular endonucleases. The present study demonstrated that p5RHH-mRNA nanoparticles provided protection from RNaseA degradation. Moreover, the transfection efficiency of p5RHH-mRNA nanoparticles was also unaffected by RNaseA treatment, which is a promising stability attribute for in vivo applications.

Recent advances in the design of non-viral delivery systems for synthetic mRNA have been reported for selective targeting to specific organs. However, the majority of these reports highlight selectivity that is restricted to liver, lung, or spleen. Previous work with p5RHH-siRNA nanoparticles has demonstrated passive permeation and prolonged residence only in regions of disrupted endothelial cell barriers but minimal uptake in liver, lung, or spleen. The restriction of the p5RHH-mRNA nanoparticles to leaky or damaged vasculature confers an additional level of safety for systemic administration as tissues with intact barrier function will not accumulate particles. To
that point, we did not observe niRFP expression in plaque free regions of the same aortas. To our knowledge this work is the first report of nanoparticle-mediated mRNA delivery to atherosclerotic plaques after systemic administration. Strikingly, we were unable to detect any niRFP transcripts in other organs of the ApoE-/- mice, nor did we detect any GFP in the organs of C57BL6/J mice after treatment with p5RHH-GFP mRNA nanoparticles. The possibility of delivering both siRNA and mRNA simultaneously to the same area with the same platform may provide robust control of protein expression, which might increase both safety and specificity for future therapeutic applications.

Other penetrating peptide-based nucleic acid delivery system have used chemically modified transportan-derived peptides such as PepFect. However, unlike p5RHH, the original PepFect complexes exhibit poor endosomal escape and required chloroquine treatment\textsuperscript{310} or conjugation of chloroquine analogs to the peptide to increase transfection efficiency\textsuperscript{311}. In addition, no reports have demonstrated the ability of PepFect to deliver mRNA payloads, despite success in plasmid DNA delivery. Furthermore, the in vivo administration of the PepFect complexes lead to accumulation in the liver or lungs, depending on the specific peptide modification\textsuperscript{312}, in contrast to p5RHH complexes that avoid the macrophage phagocytic system as shown in this and prior work\textsuperscript{242,246}.

**Conclusions**

In conclusion, we have reported a highly efficient mRNA delivery platform based on a modified cell penetrating peptide, p5RHH. p5RHH-mRNA nanoparticles
spontaneously assemble in solution without the need for filtering or removal of toxic solvents. Complexing of p5RHH with mRNA payloads attenuates its membrane lytic activity until the nanoparticles are disassembled in the acidic environment of endosomes. These nanoparticles exhibit a unique distribution after systemic administration, resulting in robust expression of the payload mRNA in atherosclerotic plaques. In addition, the flexibility of complexing any RNA with p5RHH in a simple mixing procedure may prove advantageous for both clinical and pre-clinical use.
Chapter 7
Limitations and Overall Conclusions

Study Limitations

In our examination of the effects of modified nucleotides on the regulation of miRNA switches, we utilized a variety of modified nucleotides at multiple percentages of substitution. However, this does not permit the modification of specific bases and therefore produces heterogeneity in the product transcripts. Given the enrichment of nucleotide modifications in specific regions of endogenous mRNA, the effects of transcript-wide incorporation of modified nucleotides may mask the effects produced by modification of specific loci. Further studies using a nucleotide modifying enzyme fused to an RNA-targeting Cas13 CRISPR effector, similar to reported RNA editing systems \(^{313}\), may be better able to elucidate the importance of modification location. In an attempt to test the generality of the effects observed using the miR-126-3p switches, we utilized switches with target sites for different miRNA. We only tested a few additional miRNA that were specifically selected miRNA as they were known to be expressed at a high level in various cell types. A more thorough study would have also investigated the silencing of a cognate nucleotide-modified miRNA switches by other less robustly expressed miRNA. Such data could also be used to determine the interaction between the miRNA expression level and the effect of nucleotide modification on the silencing of miRNA switches. Furthermore, testing different miRNA target sites at the 5’ UTR would also be beneficial, though our results are supported by other published studies \(^{144}\). We
also used only one 5' and 3' UTR to minimize the variability of our results. Further studies that examine these affects in different UTRs are required to further confirm the generality of our results. In addition, a more sensitive assay, such as luciferase activity, may better show the differences between the various miRNA switches. However, we reasoned that the ability to normalize the expression of the GFP reporter to an endogenous control protein, GAPDH, by immunoblotting was worth the loss in sensitivity. Finally, the expression of GFP was assayed at the 24-hour timepoint in all of the reported experiments, but translation of synthetic mRNA is often reported to peak around 6-10 hours after transfection \(^{104}\). Examining differences at earlier or later timepoints may reveal other changes in regulation caused by the nucleotide modification.

In our study we did not directly track the uptake of p5RHH-mRNA nanoparticles into endosomes. However, the results we observed after inhibition of endosomal acidification by bafilomycin are supported by previous studies using p5RHH-siRNA nanoparticles that were colocalized with endosomal markers \(^{241}\). Furthermore, we observed that not all cells that were positive for the presence of the fluorescently-labeled mRNA were also GFP positive. However, we interpreted the lack of increased percentage of GFP expressing cells after chloroquine treatment to indicate efficient endosomal escape, but this is not a direct measurement, which would require intensive microscopic analysis. While we did not detect expression of GFP either by immunoblotting or the presence of the synthetic mRNA by RT-PCR 48 hours after administration of p5RHH-GFP nanoparticles to healthy mice, tissue distribution should also be assessed at an earlier timepoint. In addition, the presence of the cargo mRNA
could be determined *in situ* by utilizing the Cy5-labelled GFP mRNA, however, the presence of the fluorescent signal would not necessarily indicate the expression of the encoded protein. In fact, previous work with fluorescently-labeled p5RHH-siRNA nanoparticles showed significant signal in the kidney after systemic administration but no apparent effect of the treatment 245.

**Overall Conclusions**

In this study we investigated the influence of modified nucleotides on the performance of miRNA switches. We found that incorporation of modified nucleotides that significantly increase translation, such as Ψ and m1Ψ, tended to decrease the miRNA-dependent regulation of miRNA switches. However, we also found that Ψ/m5C modification enabled one miRNA target site at the 3’ UTR to regulate the miRNA switch as effectively as four target sites. We also demonstrated that the effects of Ψ, Ψ/m5C, and m1Ψ are dependent on the sequence of the miRNA site but not the proportion of the number of modified nucleotides within the site. Furthermore, we found that effects of nucleotide modification are modified miRNA switches with seed complementary target sites are poorly regulated by miRNA, while placing the miRNA target site in the 5’ UTR makes the miRNA-dependent silencing largely insensitive to nucleotide modification.

We also demonstrated that p5RHH peptides spontaneously form compact nanoparticles in the presence of mRNA across a wide range of mRNA sizes. These nanoparticles are highly transfective and RNase resistant. Furthermore, they achieve robust endosomal escape with minimal cytotoxicity. Systemic delivery of p5RHH-mRNA nanoparticles generated high expression levels of the synthetic mRNA exclusively in
atherosclerotic plaque regions with no detectable expression in typical depot organs. The simple method of production and the use of only biologically compatible solutions makes p5RHH-mRNA nanoparticles an attractive platform for both pre-clinical and clinical applications.
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MEMORANDUM

TO: Hana Totary-Jain,

FROM: Farah Mouli, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 8/31/2017

PROJECT TITLE: Peptide-assisted mRNA delivery

FUNDING SOURCE: USF department, institute, center, etc.
National Institutes of Health

IACUC PROTOCOL #: R IS00004197

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 8/31/2017:

Mouse: C57BL/6 ((20g male or female)) 165

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.