COMPOSITIONS AND METHODS OF ALTERING CHOLESTEROL LEVELS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No 61/786,737, filed March 15, 2013, entitled Compositions and Methods of Altering Cholesterol Levels; U.S. Provisional Patent Application No 61/828,214, filed May 29, 2013, entitled Compositions and Methods of Altering Cholesterol Levels; U.S. Provisional Patent Application No 61/839,488, filed June 26, 2013, entitled Compositions and Methods of Altering Cholesterol Levels; U.S. Provisional Patent Application No 61/903,474, filed November 13, 2013, entitled Compositions and Methods of Altering Cholesterol Levels; and U.S. Application No. 14/135,887, filed December 20, 2013, entitled Compositions and Methods of Altering Cholesterol Levels, the contents of each of which is herein incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a filed entitled M044PCTSQLST.txt, created on March 13, 2014 which is 175,503 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention is directed to compositions and methods for modulating and/or altering cholesterol levels in an organism or altering cholesterol trafficking in an organism. In one aspect, the invention relates to RNA such as modified RNA in therapeutics. The RNA or modified RNA of the invention may encode peptides, polypeptides or multiple proteins. The RNA or modified RNA of the invention may also be used to produce polypeptides of interest. The modified RNA molecules of the invention may be mRNA and therefore be referred to as modified

mRNA. The polypeptides of interest may be used in therapeutics and/or clinical and research settings.

BACKGROUND OF THE INVENTION

[0004] High cholesterol is one of a number of risk factors for heart attack and stroke. Although poor diet and lack of excise are common causes of high cholesterolgenetic changes, such as familiar hypercholesterolemia (FH), which is caused by deficiency in LDLR, can be causes of high cholesterol. A number of cholesterol lowering drugs are currently on the market but they are not without risk or contraindications with certain conditions or other medications. Such drugs include statins, fibrates, niacin, bile acid sequestrants (resins), phytosterols, or other compounds that prevent absorption of fats, reduce absorption of cholesterol, or target genes in the cholesterol trafficking pathway.

[0005] Nucleic acid based cholesterol lowering drugs include, for example an antisense oligonucleotide inhibitor which targets ApoB-100, mipomersen, which was approved in January 2013 for the treatment of homozygous familial hypercholesterolemia (FH). In December of 2012, the FDA also approved lomitapide for the same condition.

[0006] More troubling are the liver related problems associated with cholesterol targeting drugs, particularly elevation in serum transaminases and accumulation of hepatic fat (or hepatic steatosis). For example, because of the potentially significant safety concerns surrounding mipomersen, the drug will carry a boxed warning about liver toxicity as well as requiring certification of prescribers and pharmacies, as well as documentation that the drug is being properly used with each new prescription. While mipomersen was generally effective in lowering LDL cholesterol (more than half of patients in clinical trials had more than a 20% decrease in LDL levels and in the homozygous FH trial, it reduced LDL by 24.7%), a typical FH patient has an average LDL between 400-1000mg/dL. Consequently, lowering was not likely enough in these patients. In addition, the trials were not large enough to be powered to assess cardiovascular outcomes, though cardiovascular benefit is of course the ultimate intended effect of the drug. Further, serious adverse events of cardiac disorders occurred in the mipomersen group in phase 3 trials.

[0007] The present invention addresses both the problem of elevated LDL cholesterol levels and dysregulation of hepatic function by providing nucleic acid based compounds or polynucleotides which encode a polypeptide of interest (e.g., modified mRNA or mmRNA) and which have structural and/or chemical features that avoid one or more of the problems in the art.

[0008] To this end, the inventors have shown that certain modified mRNA sequences have the potential as therapeutics with benefits beyond just evading, avoiding or diminishing the immune response. Such studies are detailed in published co-pending applications International Application PCT/US2011/046861 filed August 5, 2011 and PCT/US2011/054636 filed October 3, 2011, International Application number PCT/US2011/054617 filed October 3, 2011, the contents of which are incorporated herein by reference in their entirety.

SUMMARY OF THE INVENTION

[0009] Described herein are compositions, methods and kits RNA using RNA such as modified RNA in the treatment, prevention or diagnosis of disease and disorders associated with cholesterol and/or cholesterol trafficking.

[0010] According to the present invention, the pathways associated with cholesterol trafficking are modulated by providing one or more polypeptides (including enzymes) which alter either the concentrations of cholesterol, its processing or transport.

[0011] In one embodiment, the transport of LDL cholesterol from plasma to liver cells is increased by providing the cell with either more receptor molecules or by minimizing the destruction of the LDL receptor. In the first instance a polynucleotide, primary construct or mmRNA is provided which encodes LDL receptor. In the second instance, a mutant form of LDL receptor is encoded by the polynucleotide, primary construct or mmRNA. Such mutant LDL receptors (LDL-R or LDLR) would be deficient in some way in their binding of PCSK-9. Accordingly, a PCSK9 binding deficient LDLR would bring cholesterol into the hepatocyte.

[0012] Provided herein are polynucleotides, primary constructs and/or mmRNA which encode an LDLR mutant. In one aspect, a modified mRNA may encode a LDLR mutant which may comprise at least one amino acid mutation in a region comprising amino acids 314-393 of LDLR such as, but not limited to, SEQ ID NO:

19. In one embodiment, the region of amino acids comprises amino acids 316-339 of SEQ ID NO: 19. The modified mRNA may comprise at least one nucleoside modification such as, but not limited to, 1-methylpseudouridine. The modified mRNA may also comprise the nucleoside modification 5-methylcytosine.

[0013] Provided herein are also methods of reducing serum cholesterol in a subject comprising administering to the subject a modified mRNA may encode a LDLR mutant which may comprise at least one amino acid mutation in a region comprising amino acids 314-393 of LDLR such as, but not limited to, SEQ ID NO: 19. In one embodiment, the region of amino acids comprises amino acids 316-339 of SEQ ID NO: 19.

[0014] In one embodiment, the hepatocyte is provided with one or more polynucleotides, primary constructs, or mmRNA encoding and/or which overexpresses CYP7A1. CYP7A1 is the rate limiting enzyme for bile acid synthesis, and promotes removal of the incoming cholesterol. There are humans with CYP7A1 mutations that are associated with high plasma low-density lipoprotein (LDL) and hepatic cholesterol content, as well as deficient bile acid excretion.

[0015] In one embodiment, two polynucleotides, primary constructs, or mmRNA are delivered resulting in lower plasma cholesterol and concomitant enhanced cholesterol disposal.

[0016] In one embodiment, the one or more polynucleotides, primary constructs or mmRNA are modified in the 3'UTR to contain a microRNA binding site or seed. In this embodiment, the CYP7A1polynucleotide, having a normal half life of approximately 30 minutes, may be made transcription-dependent by miR-destabilization (specifically the ubiquitous miR122a in hepatocytes). According to this embodiment, a miR122a binding site may be incorporated into the 3'UTR of the mmRNA rendering the transcript less stable. This would allow, depending on the number of binding sites engineered into the construct, the titration of stability and therefore allow for control of expression of the encoded CYP7A1 enzyme. The polynucleotides, primary constructs primary constructs or mmRNA encoding CYP7A1 may also be useful in creating mouse models useful in proof of concept

studies and basic research. These studies would be analogous to producing dose dependent gene therapy.

[0017] In one embodiment, treatment regimes may be designed for rare diseases where patients present with CYP7A1 polymorphisms that are hyporesponsive to statins. In this instance, studies of effective compositions of the present invention may be completed quickly and based on diet-based challenges. As such the compositions of the present invention are useful in the study and treatment of disease involving cholesterol related diseases, both rare and prevalent.

[0018] In one embodiment, the compositions of the present invention may be administered along with other drug compounds. Such other drugs include specifically statins. Examples of statins include, but are not limited to, atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, simvastatin, and combinations thereof.

[0019] According to the present invention, the compositions comprising polynucleotides, primary constructs or mmRNA are useful in treating diseases such as rare non-alcoholic fatty liver disease. In treating this disorder, it is contemplated that any therapeutic that drives hepatic cholesterol to its natural sink (out of the body through biles) would have superior treatment outcomes. Consequently, it is contemplated that administration of polynucleotides, primary constructs or mmRNA encoding LDLR would increase cholesterol in the hepatocyte but that co-administration of a second polynucleotides, primary construct, or mmRNA encoding CYP7A1 would continue to drive the cholesterol out through bile thereby avoiding the fatty liver symptoms currently seen with known therapeutics.

[0020] In addition to delivering at least LDLR or a PCSK9 LDLR mutant along with CYP7A1 polynucleotides, primary constructs or mmRNAs, it is further expected that delivering an additional drug such as a statin would be very synergistic to the LDLR-CYP7A1 therapy described herein. Consequently, cholesterol excretion would be promoted, new formation would be prevented and transport from the plasma would be increased.

[0021] In one embodiment, a mix of mmRNA would be titrated along the cholesterol homeostasis pathway to promote mobilization of cholesterol out of the body.

[0022] In another embodiment, bile acid sequestrants or fat soluble vitamins may be co-administered.

[0023] It is further appreciated that certain features of the present disclosure, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the present disclosure which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

[0024] The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[0026] FIG. 1 is a schematic of a primary construct of the present invention.

[0027] FIG. 2 is a schematic of a primary construct of the present invention.

[0028] FIG. 3 shows the pathway of cholesterol trafficking in a liver cell.

[0029] FIG. 4 is a flow cytometry plot of low density lipoprotein receptor (LDLR) modified mRNA.

[0030] FIG. 5 is a flow cytometry plot of LDLR modified mRNA.

[0031] FIG. 6 is a graph of LDLR Expression. Figure 6A shows LDL Receptor Expression of cells compared to LDLR mRNA added. Figure 6B shows LDL Receptor Expression of cells post transfection. Figure 5C shows the saturation of BODIPY® labeled LRL. Figure 6D shows the binding affinity of BODIPY-LDL to cells. Figure 6E shows the total cholesterol content of each fraction.

[0032] FIG. 7 is a bioanalyzer image of LDLR modified mRNA product. Lane 1, size markers in nucleotides; Lane 2, LDLR modified mRNA.

[0033] FIG. 8 is a flow cytometry plot of 800 ng LDLR modified mRNA transfected in HEK293 cells.

[0034] FIG. 9 is a flow cytometry plot of LDLR modified mRNA transfected in HEK293 cells.

[0035] FIG. 10 shows the cholesterol level in serum. Figure 10A shows the absorbance profile of FPLC fractions from pooled LDLR knock out mice (Upper panel) and wild type mice (lower panel). Figure 10B shows the total cholesterol content of each fraction.

[0036] FIG. 11 is a flow cytometry plot of variant LDLR modified mRNA transfected in HEK293 cells.

[0037] FIG. 12 is a flow cytometry plot of variant LDLR modified mRNA transfected in HEK293 cells with or without PCSK9.

[0038] FIG. 13 is a flow cytometry plot of transfected variant LDLR modified mRNA. Figure 13A shows contour plots of the binding of BODIPY-LDL to LDLR mRNA transfected cells. Figure 13B shows the half-maximal cell association of BODIPY-LDL.

[0039] FIG. 14 shows the effect on half-life after transfection with LDLR mRNA. Figure 134 shows wild-type LDLR mRNA. Figure 14B shows a LDLR mRNA encoding a variant LDLR with 4 amino acid substitutions (N316A, E317A, D331A and Y336A). Figure 14C shows a LDLR mRNA encoding a variant LDLR with 1 amino acid substitution, Y336A. Figure 14D shows a LDLR mRNA encoding a variant LDLR with 1 amino acid substitution, E317A. Figure 14E shows a LDLR mRNA encoding a variant LDLR with 1 amino acid substitution, N316A. Figure 14F shows a LDLR mRNA encoding a variant LDLR with 1 amino acid substitution, L339D. Figure 14G shows a LDLR mRNA encoding a variant LDLR with 1 amino acid substitution, D331E.

[0040] FIG. 15 shows the effect on cell surface LDLR when the amount of PCSK is varied.

DETAILED DESCRIPTION

[0041] It is of great interest in the fields of therapeutics, diagnostics, reagents and for biological assays to be able to deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, whether *in vitro*, *in vivo, in situ* or *ex vivo*, such as to cause intracellular translation of the nucleic acid and production of an encoded polypeptide of interest. Of particular importance is the delivery and function of a non-integrative polynucleotide.

[0042] Described herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of polynucleotides encoding one or more polypeptides of interest. Also provided are systems, processes, devices and kits for the selection, design and/or utilization of the polynucleotides encoding the polypeptides of interest described herein.

[0043] According to the present invention, these polynucleotides are preferably modified as to avoid the deficiencies of other polypeptide-encoding molecules of the art. Hence these polynucleotides are referred to as modified mRNA or mmRNA.

[0044] Provided herein, in part, are polynucleotides, primary constructs and/or mmRNA encoding polypeptides of interest which have been designed to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access by the compositions, engagement with translational machinery, mRNA half-life, translation efficiency, immune evasion, protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, function and/or activity. Specifically, the polynucleotides, primary constructs and/or mmRNA of the present invention are useful in altering cholesterol levels or cholesterol trafficking in an organism, particularly human patients.

[0045] According to the present invention, the pathways associated with cholesterol trafficking are modulated by providing one or more polypeptides (including enzymes) which alter either the concentrations of cholesterol, its processing or transport.

[0046] In one embodiment, the transport of LDL cholesterol from plasma to liver cells is increased by providing the cell with either more receptor molecules or by minimizing the destruction of the LDL receptor. In the first instance a polynucleotide, primary construct or mmRNA is provided which encodes LDL

receptor. In the second instance, a mutant form of LDL receptor is encoded by the polynucleotide, primary construct or mmRNA. Such mutant LDL receptors (LDL-R or LDLR) would be deficient in some way in their binding of PCSK-9. The binding site of PCSK-9 has been previously localized to the EGF-A (or EGF-like repeat) domain of LDLR (see e.g., Kwon et al. *Molecular Basis for LDL receptor recognition by PCSK9*. PNAS. 2008 105(6), 1820-1825; the contents of which is herein incorporated by reference in its entirety). Accordingly, a PCSK9 binding deficient LDLR would bring cholesterol into the hepatocyte.

[0047] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a mutant LDLR which is deficient in binding to PCSK-9.

[0048] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a mutant LDLR which comprises at least one amino acid mutation in the PCSK-9 binding site. The mutant LDLR may comprise one, two, three, four, five, six, seven, eight, nine, ten or more than ten mutations.

[0049] In one embodiment, the mutant LDLR may comprise at least one amino acid mutation in the EGF-A domain (also known as the EGF-like repeat domain) of LDLR. As a non-limiting example, the EGF-A domain is located in a region of LDLR comprising amino acids 314-393. As another non-limiting example, the EGF-A domain is a region of SEQ ID NO: 19 comprising amino acids 314-393.

[0050] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a mutant LDLR which comprises at least one amino acid mutation in the EGF-like 1 domain. The EGF-like 1 domain may be located in a region of LDLR comprising amino acids 314-353. As a non-limiting example, the EGF-like 1 domain is a region of SEQ ID NO: 19 comprising amino acids 314-353.

[0051] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a mutant LDLR which comprises at least one amino acid mutation in the EGF-like 2 domain. The EGF-like 2 domain may be located in a region of LDLR comprising amino acids 353-393. As a non-limiting example, the EGF-like 2 domain is a region of SEQ ID NO: 19 comprising amino acids 353-393.

[0052] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a PCSK-9 binding deficient mutant LDLR. The PCSK-9 binding deficient

mutant LDLR may comprise at least one amino acid mutation in the region of SEQ ID NO: 19 comprising amino acids 314-393. As a non-limiting example, at least one mutation may be located between amino acids 314-353. As another non-limiting example, at least one mutation may be located between amino acids 315-340. As yet another non-limiting example, at least one mutation may be located between amino acids 354-393.

[0053] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a PCSK-9 binding deficient mutant LDLR comprising at least one mutation in the PCSK-9 binding region. The mutation may be located in the region of SEQ ID NO: 19 comprising amino acids 314-393. As non-limiting examples of regions of mutations, the mutation may be located in the region of 314-353, 315-340 and 354-393. As another non-limiting example, the mutation may be at position 316, 317, 331, 336 or 339. As yet another non-limiting example, the mutant LDLR may comprise a mutation at position 316, 317, 331 and 336.

[0054] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a PCSK-9 binding deficient mutant LDLR comprising at least one mutation at an amino acid position such as, but not limited to, 316, 317, 331, 336 and/or 339. As a non-limiting example, the PCSK-9 binding deficient mutant LDLR may comprise at least one of the mutations N316A, E317A, D331A, D331E, Y336A and/or L339D where "N316A" means Asparagine at position 316 is replaced with Alanine. As another non-limiting example, the PCSK-9 binding deficient mutant LDLR may comprise the mutations N316A, E317A, D331A and Y336A.

[0055] In one embodiment, a polynucleotide, primary construct or mmRNA may encode a PCSK-9 binding deficient mutant LDLR comprising four mutations at amino acid positions such as, but not limited to, 316, 317, 331, 336 and/or 339. As a non-limiting example, the PCSK-9 binding deficient mutant LDLR may comprise any four of the mutations such as N316A, E317A, D331A, D331E, Y336A and/or L339D where "N316A" means Asparagine at position 316 is replaced with Alanine. As another non-limiting example, the PCSK-9 binding deficient mutant LDLR may comprise the mutations N316A, E317A, D331A and Y336A.

[0056] In one embodiment, the hepatocyte is provided with one or more polynucleotides, primary constructs, or mmRNA encoding and/or which overexpresses CYP7A1. CYP7A1 is the rate limiting enzyme for bile acid synthesis, and promotes removal of the incoming cholesterol. There are humans with CYP7A1 mutations that are associated with high plasma low-density lipoprotein (LDL) and hepatic cholesterol content, as well as deficient bile acid excretion.

[0057] In one embodiment, two polynucleotides, primary constructs, or mmRNA are delivered resulting in lower plasma cholesterol and concomitant enhanced cholesterol disposal.

[0058] In one embodiment, the one or more polynucleotides, primary constructs or mmRNA are modified in the 3'UTR to contain a microRNA binding site or seed. In this embodiment, the CYP7A1 polynucleotide, having a normal half life of approximately 30 minutes, may be made transcription-dependent by miR-destabilization (specifically the ubiquitous miR122a in hepatocytes). According to this embodiment, a miR122a binding site may be incorporated into the 3'UTR of the mmRNA rendering the transcript less stable. This would allow, depending on the number of binding sites engineered into the construct, the titration of stability and therefore allow for control of expression of the encoded CYP7A1 enzyme. The polynucleotides, primary constructs primary constructs or mmRNA encoding CYP7A1 may also be useful in creating mouse models useful in proof of concept studies and basic research. These studies would be analogous to producing dose dependent gene therapy.

[0059] In one embodiment, treatment regimes may be designed for rare diseases where patients present with CYP7A1 polymorphisms that are hyporesponsive to statins. In this instance, studies of effective compositions of the present invention may be completed quickly and based on diet-based challenges. As such the compositions of the present invention are useful in the study and treatment of disease involving cholesterol related diseases, both rare and prevalent.

[0060] In one embodiment, the compositions of the present invention may be administered along with other drug compounds. Such other drugs include specifically statins. Examples of statins include, but are not limited to, atorvastatin, cerivastatin,

fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, simvastatin, and combinations thereof.

[0061] According to the present invention, the compositions comprising polynucleotides, primary constructs or mmRNA are useful in treating diseases such as rare non-alcoholic fatty liver disease. In treating this disorder, it is contemplated that any therapeutic that drives hepatic cholesterol to its natural sink (out of the body through biles) would have superior treatment outcomes. Consequently, it is contemplated that administration of polynucleotides, primary constructs or mmRNA encoding LDLR would increase cholesterol in the hepatocyte but that co-administration of a second polynucleotides, primary construct, or mmRNA encoding CYP7A1 would continue to drive the cholesterol out through bile thereby avoiding the fatty liver symptoms currently seen with known therapeutics.

[0062] In addition to delivering at least LDLR or a PCSK9 LDLR mutant along with CYP7A1 polynucleotides, primary constructs or mmRNAs, it is further expected that delivering an additional drug such as a statin would be very synergistic to the LDLR-CYP7A1 therapy described herein. Consequently, cholesterol excretion would be promoted, new formation would be prevented and transport from the plasma would be increased.

[0063] In one embodiment, a mix of mmRNA would be titrated along the cholesterol homeostasis pathway to promote mobilization of cholesterol out of the body.

[0064] In another embodiment, bile acid sequestrants or fat soluble vitamins may be co-administered.

[0065] It is further appreciated that certain features of the present disclosure, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the present disclosure which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

I. Compositions of the Invention (mmRNA)

[0066] The present invention provides nucleic acid molecules, specifically polynucleotides, primary constructs and/or mmRNA which encode one or more

polypeptides of interest. The term "nucleic acid," in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides. These polymers are often referred to as polynucleotides. Exemplary nucleic acids or polynucleotides of the invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2′-amino-LNA having a 2′-amino functionalization, and 2′-amino- α-LNA having a 2′-amino functionalization) or hybrids thereof.

[0067] In preferred embodiments, the nucleic acid molecule is a messenger RNA (mRNA). As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide, such as a synthetic polynucleotide, which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest *in vitro, in vivo, in situ* or *ex vivo*.

[0068] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5′UTR, a 3′UTR, a 5′ cap and a poly-A tail. Building on this wild type modular structure, the present invention expands the scope of functionality of traditional mRNA molecules by providing polynucleotides or primary RNA constructs which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the reprogrammming polynucleotides including, in some embodiments, the lack of a substantial induction of the innate immune response of a cell into which the polynucleotide is introduced. As such, modified mRNA molecules of the present invention, such as synthetic modified mRNA molecules, are termed "mmRNA." As used herein, a "structural" feature or modification is one in which two or more linked nucleotides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide, primary construct or mmRNA without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the

polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

mmRNA Architecture

[0069] The mmRNA of the present invention are distinguished from wild type mRNA in their functional and/or structural design features which serve to, as evidenced herein, overcome existing problems of effective polypeptide production using nucleic acid-based therapeutics.

[0070] Figure 1 shows a representative polynucleotide primary construct **100** of the present invention. As used herein, the term "primary construct" or "primary mRNA construct" refers to a polynucleotide transcript which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Primary constructs may be polynucleotides of the invention. When structurally or chemically modified, the primary construct may be referred to as an mmRNA.

[0071] Returning to FIG. **1**, the primary construct **100** here contains a first region of linked nucleotides **102** that is flanked by a first flanking region **104** and a second flaking region **106**. As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region." This first region may include, but is not limited to, the encoded polypeptide of interest. The polypeptide of interest may comprise at its 5' terminus one or more signal sequences encoded by a signal sequence region **103**. The flanking region **104** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5′ UTRs sequences. The flanking region **104** may also comprise a 5′ terminal cap **108**. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3′ UTRs. The flanking region **106** may also comprise a 3′ tailing sequence **110**.

[0072] Bridging the 5′ terminus of the first region **102** and the first flanking region **104** is a first operational region **105**. Traditionally this operational region comprises a

Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[0073] Bridging the 3′ terminus of the first region **102** and the second flanking region **106** is a second operational region **107**. Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Stop codon. According to the present invention, multiple serial stop codons may also be used.

[0074] Figure **2** shows a representative polynucleotide primary construct **130** of the present invention. Polynucleotide primary construct refers to a polynucleotide transcript which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Non-limiting examples of polypeptides of interest and polynucleotides encoding polypeptide of interest are described in Table 6 of U.S. Provisional Patent Application No 61/618,862, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Biologics; U.S. Provisional Patent Application No 61/681,645, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Biologics; U.S. Provisional Patent Application No 61/737,130, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Biologics; International Application No PCT/US2013/030062, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Biologics and Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/618,866, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Antibodies; U.S. Provisional Patent Application No 61/681,647, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Antibodies; U.S. Provisional Patent Application No 61/737,134, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Antibodies; International Application No PCT/US2013/030063, filed March 9, 2013, entitled Modified Polynucleotides; U.S. Provisional Patent Application No 61/618,868, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/681,648, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/737,135,

filed December 14, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/618,870, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/681,649, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/737,139, filed December 14, 2012, Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/618,873, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/681,650, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/737,147, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; International Application No. PCT/US2013/030064, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/618,878, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; U.S. Provisional Patent Application No 61/681,654, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; U.S. Provisional Patent Application No 61/737,152, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; International Application No PCT/US2013/030059, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Membrane Proteins; U.S. Provisional Patent Application No 61/618,885, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/681,658, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/737,155, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; International Application No. PCT/US2013/030066, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/618,896, filed

April 2, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/668,157, filed July 5, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/681,661, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/737,160, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/618,911, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/681,667, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/737,168, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; International Application No. PCT/US2013/030067, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/618,922, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/681,675, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/737,174, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins; International Application No. PCT/US2013/030060, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/618,935, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,687, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,184, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; International Application No. PCT/US2013/030061, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/618,945, filed April 2, 2012, entitled Modified Polynucleotides for

the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,696, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,191, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/618,953, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,704, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,203, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; International Application No. PCT/US2013/031821, filed March 15, 2013, entitled In Vivo Production of Proteins; U.S. Provisional Patent Application No 61/681,720, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Cosmetic Proteins and Peptides; U.S. Provisional Patent Application No 61/737,213, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Cosmetic Proteins and Peptides; International Application No. PCT/US2013/030068, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Cosmetic Proteins and Peptides; U.S. Provisional Patent Application No. 61/681,742, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Oncology-Related Proteins and Peptides and International Application No. PCT/US2013/030070, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Oncology-Related Proteins and Peptides, and, the contents of each of which are incorporated herein by reference in their entirety.

[0075] Returning to FIG. **2**, the polynucleotide primary construct **130** here contains a first region of linked nucleotides **132** that is flanked by a first flanking region **134** and a second flaking region **136**. As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region." This first region may include, but is not limited to, the encoded polypeptide of interest. In one aspect, the first region **132** may include, but is not limited to, the open reading frame

encoding at least one polypeptide of interest. The open reading frame may be codon optimized in whole or in part. The flanking region **134** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5′ UTRs sequences which may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences, TERZAK™ sequences and translation control sequences. The flanking region **134** may also comprise a 5′ terminal cap **138**. The 5′ terminal capping region **138** may include a naturally occurring cap, a synthetic cap or an optimized cap. Non-limiting examples of optimized caps include the caps taught by Rhoads in US Patent No. US7074596 and International Patent Publication No. WO2008157668, WO2009149253 and WO2013103659. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3′ UTRs. The second flanking region **136** may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences and translation control sequences. After the second flanking region **136** the polynucleotide primary construct may comprise a 3′ tailing sequence **140**. The 3' tailing sequence **140** may include a synthetic tailing region **142** and/or a chain terminating nucleoside **144**. Non-liming examples of a synthetic tailing region include a polyA sequence, a polyC sequence, a polyA-G quartet. Non-limiting examples of chain terminating nucleosides include 2'-O methyl, F and locked nucleic acids (LNA).

[0076] Bridging the 5′ terminus of the first region **132** and the first flanking region **134** is a first operational region **144**. Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[0077] Bridging the 3′ terminus of the first region **132** and the second flanking region **136** is a second operational region **146**. Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Stop codon. According to the present invention, multiple serial stop codons may also be used.

[0078] Generally, the shortest length of the first region of the primary construct of the present invention can be the length of a nucleic acid sequence that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[0079] Generally, the length of the first region encoding the polypeptide of interest of the present invention is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region."

[0080] In some embodiments, the polynucleotide, primary construct, or mmRNA includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000

to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[0081] According to the present invention, the first and second flanking regions may range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[0082] According to the present invention, the tailing sequence may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the tailing region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides and 160 nucleotides are functional.

[0083] According to the present invention, the capping region may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[0084] According to the present invention, the first and second operational regions may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length and may comprise, in addition to a Start and/or Stop codon, one or more signal and/or restriction sequences.

Cyclic mmRNA

[0085] According to the present invention, a primary construct or mmRNA may be cyclized, or concatemerized, to generate a translation competent molecule to assist interactions between poly-A binding proteins and 5′-end binding proteins. The

mechanism of cyclization or concatemerization may occur through at least 3 different routes: 1) chemical, 2) enzymatic, and 3) ribozyme catalyzed. The newly formed 5′-/3′-linkage may be intramolecular or intermolecular.

[0086] In the first route, the 5′-end and the 3′-end of the nucleic acid contain chemically reactive groups that, when close together, form a new covalent linkage between the 5′-end and the 3′-end of the molecule. The 5′-end may contain an NHS-ester reactive group and the 3′-end may contain a 3′-amino-terminated nucleotide such that in an organic solvent the 3′-amino-terminated nucleotide on the 3′-end of a synthetic mRNA molecule will undergo a nucleophilic attack on the 5′-NHS-ester moiety forming a new 5′-/3′-amide bond.

[0087] In the second route, T4 RNA ligase may be used to enzymatically link a 5′-phosphorylated nucleic acid molecule to the 3′-hydroxyl group of a nucleic acid forming a new phosphorodiester linkage. In an example reaction, 1μg of a nucleic acid molecule is incubated at 37°C for 1 hour with 1-10 units of T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The ligation reaction may occur in the presence of a split oligonucleotide capable of base-pairing with both the 5′- and 3′- region in juxtaposition to assist the enzymatic ligation reaction.

[0088] In the third route, either the 5′-or 3′-end of the cDNA template encodes a ligase ribozyme sequence such that during *in vitro* transcription, the resultant nucleic acid molecule can contain an active ribozyme sequence capable of ligating the 5′-end of a nucleic acid molecule to the 3′-end of a nucleic acid molecule. The ligase ribozyme may be derived from the Group I Intron, Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment). The ribozyme ligase reaction may take 1 to 24 hours at temperatures between 0 and 37°C.

mmRNA Multimers

[0089] According to the present invention, multiple distinct polynucleotides, primary constructs or mmRNA may be linked together through the 3′-end using nucleotides which are modified at the 3′-terminus. Chemical conjugation may be used to control the stoichiometry of delivery into cells. For example, the glyoxylate

cycle enzymes, isocitrate lyase and malate synthase, may be supplied into HepG2 cells at a 1:1 ratio to alter cellular fatty acid metabolism. This ratio may be controlled by chemically linking polynucleotides, primary constructs or mmRNA using a 3′-azido terminated nucleotide on one polynucleotide, primary construct or mmRNA species and a C5-ethynyl or alkynyl-containing nucleotide on the opposite polynucleotide, primary construct or mmRNA species. The modified nucleotide is added post-transcriptionally using terminal transferase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. After the addition of the 3′-modified nucleotide, the two polynucleotide, primary construct or mmRNA species may be combined in an aqueous solution, in the presence or absence of copper, to form a new covalent linkage via a click chemistry mechanism as described in the literature.

[0090] In another example, more than two polynucleotides may be linked together using a functionalized linker molecule. For example, a functionalized saccharide molecule may be chemically modified to contain multiple chemical reactive groups (SH-, NH_2-, N_3, etc…) to react with the cognate moiety on a 3′-functionalized mRNA molecule (i.e., a 3′-maleimide ester, 3′-NHS-ester, alkynyl). The number of reactive groups on the modified saccharide can be controlled in a stoichiometric fashion to directly control the stoichiometric ratio of conjugated polynucleotide, primary construct or mmRNA.

mmRNA Conjugates and Combinations

[0091] In order to further enhance protein production, primary constructs or mmRNA of the present invention can be designed to be conjugated to other polynucleotides, dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases, proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody,

that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell, hormones and hormone receptors, non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, or a drug.

[0092] Conjugation may result in increased stability and/or half life and may be particularly useful in targeting the polynucleotides, primary constructs or mmRNA to specific sites in the cell, tissue or organism.

[0093] According to the present invention, the mmRNA or primary constructs may be administered with, or further encode one or more of RNAi agents, siRNAs, shRNAs, miRNAs, miRNA binding sites, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers or vectors, and the like.

Bifunctional mmRNA

[0094] In one embodiment of the invention are bifunctional polynucleotides (e.g., bifunctional primary constructs or bifunctional mmRNA). As the name implies, bifunctional polynucleotides are those having or capable of at least two functions. These molecules may also by convention be referred to as multi-functional.

[0095] The multiple functionalities of bifunctional polynucleotides may be encoded by the RNA (the function may not manifest until the encoded product is translated) or may be a property of the polynucleotide itself. It may be structural or chemical. Bifunctional modified polynucleotides may comprise a function that is covalently or electrostatically associated with the polynucleotides. Further, the two functions may be provided in the context of a complex of a mmRNA and another molecule.

[0096] Bifunctional polynucleotides may encode peptides which are anti-proliferative. These peptides may be linear, cyclic, constrained or random coil. They may function as aptamers, signaling molecules, ligands or mimics or mimetics thereof. Anti-proliferative peptides may, as translated, be from 3 to 50 amino acids in length. They may be 5-40, 10-30, or approximately 15 amino acids long. They may be single chain, multichain or branched and may form complexes, aggregates or any multi-unit structure once translated.

Noncoding Polynucleotides and Primary Constructs

[0097] As described herein, provided are polynucleotides and primary constructs having sequences that are partially or substantially not translatable, e.g., having a noncoding region. Such noncoding region may be the "first region" of the primary construct. Alternatively, the noncoding region may be a region other than the first region. Such molecules are generally not translated, but can exert an effect on protein production by one or more of binding to and sequestering one or more translational machinery components such as a ribosomal protein or a transfer RNA (tRNA), thereby effectively reducing protein expression in the cell or modulating one or more pathways or cascades in a cell which in turn alters protein levels. The polynucleotide or primary construct may contain or encode one or more long noncoding RNA (lncRNA, or lincRNA) or portion thereof, a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA).

Polypeptides of interest

[0098] According to the present invention, the primary construct is designed to encode one or more polypeptides of interest or fragments thereof. A polypeptide of interest may include, but is not limited to, whole polypeptides, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more nucleic acids, a plurality of nucleic acids, fragments of nucleic acids or variants of any of the aforementioned. As used herein, the term "polypeptides of interest" refers to any polypeptides which are selected to be encoded in the primary construct of the present invention. As used herein, "polypeptide" means a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multichain polypeptides such as antibodies or insulin and

may be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

[0099] The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50% identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

[00100] In some embodiments "variant mimics" are provided. As used herein, the term "variant mimic" is one which contains one or more amino acids which would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, e.g., phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

[00101] "Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

[00102] By "homologs" as it applies to polypeptide sequences means the corresponding sequence of other species having substantial identity to a second sequence of a second species.

[00103] "Analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid

residues that still maintain one or more of the properties of the parent or starting polypeptide.

[00104] The present invention contemplates several types of compositions which are polypeptide based including variants and derivatives. These include substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

[00105] As such, mmRNA encoding polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this invention. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

[00106] "Substitutional variants" when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

[00107] As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine,

valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

[00108] "Insertional variants" when referring to polypeptides are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid.

[00109] "Deletional variants" when referring to polypeptides are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

[00110] "Covalent derivatives" when referring to polypeptides include modifications of a native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing agent, and/or post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

[00111] Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the polypeptides produced in accordance with the present invention.

[00112] Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

[00113] "Features" when referring to polypeptides are defined as distinct amino acid sequence-based components of a molecule. Features of the polypeptides encoded by the mmRNA of the present invention include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

[00114] As used herein when referring to polypeptides the term "surface manifestation" refers to a polypeptide based component of a protein appearing on an outermost surface.

[00115] As used herein when referring to polypeptides the term "local conformational shape" means a polypeptide based structural manifestation of a protein which is located within a definable space of the protein.

[00116] As used herein when referring to polypeptides the term "fold" refers to the resultant conformation of an amino acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds include beta sheets and alpha helices. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed in this way include hydrophobic and hydrophilic pockets, and the like.

[00117] As used herein the term "turn" as it relates to protein conformation means a bend which alters the direction of the backbone of a peptide or polypeptide and may involve one, two, three or more amino acid residues.

[00118] As used herein when referring to polypeptides the term "loop" refers to a structural feature of a polypeptide which may serve to reverse the direction of the backbone of a peptide or polypeptide. Where the loop is found in a polypeptide and only alters the direction of the backbone, it may comprise four or more amino acid residues. Oliva et al. have identified at least 5 classes of protein loops (J. Mol Biol 266 (4): 814-830; 1997). Loops may be open or closed. Closed loops or "cyclic" loops may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids between the bridging moieties. Such bridging moieties may comprise a cysteine-cysteine bridge (Cys-Cys) typical in polypeptides having disulfide bridges or alternatively bridging moieties may be non-protein based such as the dibromozylyl agents used herein.

[00119] As used herein when referring to polypeptides the term "half-loop" refers to a portion of an identified loop having at least half the number of amino acid resides as the loop from which it is derived. It is understood that loops may not always contain an even number of amino acid residues. Therefore, in those cases where a loop contains or is identified to comprise an odd number of amino acids, a half-loop of the odd-numbered loop will comprise the whole number portion or next whole number portion of the loop (number of amino acids of the loop/2+/-0.5 amino acids). For example, a loop identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4).

[00120] As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

[00121] As used herein when referring to polypeptides the term "half-domain" means a portion of an identified domain having at least half the number of amino acid resides as the domain from which it is derived. It is understood that domains may not always contain an even number of amino acid residues. Therefore, in those cases where a domain contains or is identified to comprise an odd number of amino acids, a

half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of amino acids of the domain/2+/-0.5 amino acids). For example, a domain identified as a 7 amino acid domain could produce half-domains of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the amino acids that comprise any of the domain types herein need not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino acids may fold structurally to produce a domain, half-domain or subdomain).

[00122] As used herein when referring to polypeptides the terms "site" as it pertains to amino acid based embodiments is used synonymously with "amino acid residue" and "amino acid side chain." A site represents a position within a peptide or polypeptide that may be modified, manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention.

[00123] As used herein the terms "termini" or "terminus" when referring to polypeptides refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide based molecules of the present invention may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins of the invention are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

[00124] Once any of the features have been identified or defined as a desired component of a polypeptide to be encoded by the primary construct or mmRNA of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or

duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

[00125] Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis. The resulting modified molecules may then be tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

[00126] According to the present invention, the polypeptides may comprise a consensus sequence which is discovered through rounds of experimentation. As used herein a "consensus" sequence is a single sequence which represents a collective population of sequences allowing for variability at one or more sites.

[00127] As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest of this invention. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of about 20, about 30, about 40, about 50, or about 100 amino acids which are about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 100% identical to any of the sequences described herein can be utilized in accordance with the invention. In certain embodiments, a polypeptide to be utilized in accordance with the invention includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

Encoded Polypeptides

[00128] The polynucleotides, primary constructs or mmRNA of the present invention may be designed to encode polypeptides of interest such as pepetides and proteins.

[00129] In one embodimentprimary constructs or mmRNA may encode variant polypeptides which have a certain identity with a reference polypeptide sequence. As used herein, a "reference polypeptide sequence" refers to a starting polypeptide sequence. Reference sequences may be wild type sequences or any sequence to which reference is made in the design of another sequence. A "reference polypeptide sequence" may be any enconding LDLR and/or CYP7a1 or variants thereof.

[00130] The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between peptides, as determined by the number of matches between strings of two or more amino acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

[00131] In some embodiments, the polypeptide variant may have the same or a similar activity as the reference polypeptide. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite

(Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.) Other tools are described herein, specifically in the definition of "Identity."

[00132] Default parameters in the BLAST algorithm include, for example, an expect threshold of 10, Word size of 28, Match/Mismatch Scores 1, -2, Gap costs Linear. Any filter can be applied as well as a selection for species specific repeats, e.g., Homo sapiens.

Peptides

[00133] The primary constructs or mmRNA disclosed herein, may encode one or more validated or "in testing" proteins or peptides.

[00134] According to the present invention, one or more proteins or peptides currently being marketed or in development may be encoded by the polynucleotides, primary constructs or oncology-related mmRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation into the primary constructs or mmRNA of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and selectivity of the construct designs.

[00135] The polynucleotides, primary constructs and/or mmRNA may alter a biological and/or physiolocial process and/or compound such as, but not limited to, altering (e.g., slowing) the progression of a disease and/or disorder, reduce cholesterol and/or low-density lipoprotein (LDL) cholesterol, improve Crigler-Najjar syndrome, restore hepcidin and/or hemochromatosis type 2 function to regulate iron uptake, restore bile acid metabolism, reduce coronary heart disease risk for familial hypercholesterolemia and prevent hyperkeratotic plaques and corneal clouding which may heal hyperkeratotic plaques on the hands and/or feet.

[00136] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be used to express a polypeptide in cells or tissues for the purpose of replacing the protein produced from a deleted or mutated gene.

[00137] Further, the polynucleotides, primary constructs or mmRNA of the invention may be used to treat metabolic disorders related to rare liver diseases and/or disorders.

Flanking Regions: Untranslated Regions (UTRs)

[00138] Untranslated regions (UTRs) of a gene are transcribed but not translated. The 5′UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3′UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides, primary constructs and/or mmRNA of the present invention to enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites.

5′ UTR and Translation Initiation

[00139] Natural 5′UTRs bear features which play roles in for translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5′UTR also have been known to form secondary structures which are involved in elongation factor binding.

[00140] By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of the polynucleotides, primary constructs or mmRNA of the invention. For example, introduction of 5′ UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, could be used to enhance expression of a nucleic acid molecule, such as a mmRNA, in hepatic cell lines or liver. Likewise, use of 5′ UTR from other tissue-specific mRNA to improve expression in that tissue is possible – for muscle (MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (Tie-1, CD36), for myeloid

cells (C/EBP, AML1, G-CSF, GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (CD45, CD18), for adipose tissue (CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (SP-A/B/C/D).

[00141] Other non-UTR sequences may be incorporated into the 5′ (or 3′ UTR) UTRs. For example, introns or portions of introns sequences may be incorporated into the flanking regions of the polynucleotides, primary constructs or mmRNA of the invention. Incorporation of intronic sequences may increase protein production as well as mRNA levels.

[00142] The 5'UTR may selected for use in the present invention may be a structured UTR such as, but not limited to, 5'UTRs to control translation. As a non-limiting example, a structured 5'UTR may be beneficial when using any of the terminal modifications described in copending U.S. Provisional Application No. 61/758,921 filed January 31, 2013, entitled Differential Targeting Using RNA Constructs; U.S. Provisional Application No. 61/781,139 filed March 14, 2013, entitled Differential Targeting Using RNA Constructs; U.S. Provisional Application No. 61/729,933, filed November 26, 2012 entitled Terminally Optimized RNAs; U.S. Provisional Application No 61/737,224 filed December 14, 2012 entitled Terminally Optimized RNAs and U.S. Provisional Application No 61/829,359 filed May 31, 2013 entitled Terminally Optimized RNAs; each of which is herein incorporated by reference in their entirety.

3′ UTR and the AU Rich Elements

[00143] 3′UTRs are known to have stretches of Adenosines and Uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger,

whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

[00144] Introduction, removal or modification of 3′ UTR AU rich elements (AREs) can be used to modulate the stability of polynucleotides, primary constructs or mmRNA of the invention. When engineering specific polynucleotides, primary constructs or mmRNA, one or more copies of an ARE can be introduced to make polynucleotides, primary constructs or mmRNA of the invention less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using polynucleotides, primary constructs or mmRNA of the invention and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hr, 12 hr, 24 hr, 48 hr, and 7 days post-transfection.

Incorporating microRNA Binding Sites

[00145] microRNAs (or miRNA) are 19-25 nucleotide long noncoding RNAs that bind to the 3′UTR of nucleic acid molecules and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. The polynucleotides, primary constructs or mmRNA of the invention may comprise one or more microRNA target sequences, microRNA seqences, microRNA binding sites, or microRNA seeds. Such sequences may correspond to any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, or those listed in Table 7 of co-pending application USSN 61/758,921 filed January 31, 2013 (Attorney Docket Number 2030.1039), the contents of which are incorporated herein by reference in their entirety.

[00146] A microRNA sequence comprises a "seed" region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-

Crick complementarity to the miRNA target sequence. A microRNA seed may comprise positions 2-8 or 2-7 of the mature microRNA. In some embodiments, a microRNA seed may comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. In some embodiments, a microRNA seed may comprise 6 nucleotides (e.g., nucleotides 2-7 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked byan adenine (A) opposed to microRNA position 1. See for example, Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105. The bases of the microRNA seed have complete complementarity with the target sequence. By engineering microRNA target sequences into the 3′UTR of polynucleotides, primary constructs or mmRNA of the invention one can target the molecule for degradation or reduced translation, provided the microRNA in question is available. This process will reduce the hazard of off target effects upon nucleic acid molecule delivery. Identification of microRNA, microRNA target regions, and their expression patterns and role in biology have been reported (Bonauer et al., Curr Drug Targets 2010 11:943-949; Anand and Cheresh Curr Opin Hematol 2011 18:171-176; Contreras and Rao Leukemia 2012 26:404-413 (2011 Dec 20. doi: 10.1038/leu.2011.356); Bartel Cell 2009 136:215-233; Landgraf et al, Cell, 2007 129:1401-1414).

[00147] For example, if the nucleic acid molecule is an mRNA and is not intended to be delivered to the liver but ends up there, then miR-122, a microRNA abundant in liver, can inhibit the expression of the gene of interest if one or multiple target sites of miR-122 are engineered into the 3′UTR of the polynucleotides, primary constructs or mmRNA. Introduction of one or multiple binding sites for different microRNA can be engineered to further decrease the longevity, stability, and protein translation of a polynucleotides, primary constructs or mmRNA.

[00148] As used herein, the term “microRNA site” refers to a microRNA target site or a microRNA recognition site, or any nucleotide sequence to which a microRNA binds or associates. It should be understood that “binding” may follow traditional

Watson-Crick hybridization rules or may reflect any stable association of the microRNA with the target sequence at or adjacent to the microRNA site.

[00149] Conversely, for the purposes of the polynucleotides, primary constructs or mmRNA of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they naturally occur in order to increase protein expression in specific tissues. For example, miR-122 binding sites may be removed to improve protein expression in the liver. Regulation of expression in multiple tissues can be accomplished through introduction or removal or one or several microRNA binding sites.

[00150] Examples of tissues where microRNA are known to regulate mRNA, and thereby protein expression, include, but are not limited to, liver (miR-122), muscle (miR-133, miR-206, miR-208), endothelial cells (miR-17-92, miR-126), myeloid cells (miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27), adipose tissue (let-7, miR-30c), heart (miR-1d, miR-149), kidney (miR-192, miR-194, miR-204), and lung epithelial cells (let-7, miR-133, miR-126). MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 2011 18:171-176). In the polynucleotides, primary constructs or mmRNA of the invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the polynucleotides, primary constructs or mmRNA expression to biologically relevant cell types or to the context of relevant biological processes.

[00151] Lastly, through an understanding of the expression patterns of microRNA in different cell types, polynucleotides, primary constructs or mmRNA can be engineered for more targeted expression in specific cell types or only under specific biological conditions. Through introduction of tissue-specific microRNA binding sites, polynucleotides, primary constructs or mmRNA could be designed that would be optimal for protein expression in a tissue or in the context of a biological condition.

[00152] Transfection experiments can be conducted in relevant cell lines, using engineered polynucleotides, primary constructs or mmRNA and protein production can be assayed at various time points post-transfection. For example, cells can be

transfected with different microRNA binding site-engineering polynucleotides, primary constructs or mmRNA and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hr, 12 hr, 24 hr, 48 hr, 72 hr and 7 days post-transfection. *In vivo* experiments can also be conducted using microRNA-binding site-engineered molecules to examine changes in tissue-specific expression of formulated polynucleotides, primary constructs or mmRNA.

[00153] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may comprise at least one miR sequence or variant thereof. A non-exhaustive listing of miR sequences for use in polynucleotides, primary constructs and/or mmRNA is described in Table 9 of co-pending International Patent Application No. PCT/US13/62531 (M037.20), the contents of which are herein incorporated by reference in its entirety.

[00154] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may comprise at least one miR sequence that bind and inhibit the untranslated region of HMG-CoA reductase or PCSK9. Non-limiting examples of the miR sequences that bind and inhibit the untranslated region of HMG-CoA reductase or PCSK9 are described in International Patent Publication No. WO2013154766, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the polynucleotides, primary constructs and/or mmRNA may comprise a miR sequence that comprises miR-520d-5p, miR-224 or variants thereof (see e.g., International Patent Publication No. WO2013154766, the contents of which are herein incorporated by reference in its entirety). As another non-limiting example, the polynucleotides, primary constructs and/or mmRNA may comprise a miR sequence that comprises or encodes SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 10 and/or SEQ ID NO: 11 of International Patent Publication No. WO2013154766, the contents of which are herein incorporated by reference in its entirety. As yet another non-limiting example, the polynucleotides, primary constructs and/or mmRNA may comprise a miR sequence that comprises miR-224 or variants thereof (see e.g., International Patent Publication No. WO2013154766, the contents of which are herein incorporated by reference in its entirety). As another non-limiting example, the polynucleotides, primary constructs and/or mmRNA may comprise a

miR sequence that comprises miR-520d-5p and miR-224 or variants thereof (see e.g., International Patent Publication No. WO2013154766, the contents of which are herein incorporated by reference in its entirety).

5′ Capping

[00155] The 5′ cap structure of an mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsibile for mRNA stability in the cell and translation competency through the association of CBP with poly(A) binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5′ proximal introns removal during mRNA splicing.

[00156] Endogenous mRNA molecules may be 5′-end capped generating a 5′-ppp-5′-triphosphate linkage between a terminal guanosine cap residue and the 5′-terminal transcribed sense nucleotide of the mRNA molecule. This 5′-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5′ end of the mRNA may optionally also be 2′-O-methylated. 5′-decapping through hydrolysis and cleavage of the guanylate cap structure may target a nucleic acid molecule, such as an mRNA molecule, for degradation.

[00157] Modifications to the polynucleotides, primary constructs, and mmRNA of the present invention may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5′-ppp-5′ phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with α-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5′-ppp-5′ cap. Additional modified guanosine nucleotides may be used such as α-methyl-phosphonate and seleno-phosphate nucleotides.

[00158] Additional modifications include, but are not limited to, 2′-O-methylation of the ribose sugars of 5′-terminal and/or 5′-anteterminal nucleotides of the mRNA (as mentioned above) on the 2′-hydroxyl group of the sugar ring. Multiple distinct 5′-cap

structures can be used to generate the 5′-cap of a nucleic acid molecule, such as an mRNA molecule.

[00159] Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5′-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/linked to a nucleic acid molecule.

[00160] For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5′-5′-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3′-O-methyl group (i.e., N7,3′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine (m^7G-3′mppp-G; which may equivaliently be designated 3′ O-Me-m7G(5')ppp(5')G). The 3′-O atom of the other, unmodified, guanine becomes linked to the 5′-terminal nucleotide of the capped nucleic acid molecule (e.g. an mRNA or mmRNA). The N7- and 3′-O-methlyated guanine provides the terminal moiety of the capped nucleic acid molecule (e.g. mRNA or mmRNA).

[00161] Another exemplary cap is mCAP, which is similar to ARCA but has a 2′-O-methyl group on guanosine (i.e., N7,2′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine, m^7Gm-ppp-G).

[00162] While cap analogs allow for the concomitant capping of a nucleic acid molecule in an in vitro transcription reaction, up to 20% of transcripts remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5′-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

[00163] Polynucleotides, primary constructs and mmRNA of the invention may also be capped post-transcriptionally, using enzymes, in order to generate more authentic 5′-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., of the prior art, or which outperforms

the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5′cap structures of the present invention are those which, among other things, have enhanced binding of cap binding proteins, increased half life, reduced susceptibility to 5′ endonucleases and/or reduced 5′decapping, as compared to synthetic 5′cap structures known in the art (or to a wild-type, natural or physiological 5′cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2′-O-methyltransferase enzyme can create a canonical 5′-5′-triphosphate linkage between the 5′-terminal nucleotide of an mRNA and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5′-terminal nucleotide of the mRNA contains a 2′-O-methyl. Such a structure is termed the Cap1 structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5′cap analog structures known in the art. Cap structures include 7mG(5')ppp(5')N,pN2p (cap 0), 7mG(5')ppp(5')NlmpNp (cap 1), and 7mG(5')-ppp(5')NlmpN2mp (cap 2).

[00164] Because the polynucleotides, primary constructs or mmRNA may be capped post-transcriptionally, and because this process is more efficient, nearly 100% of the polynucleotides, primary constructs or mmRNA may be capped. This is in contrast to ~80% when a cap analog is linked to an mRNA in the course of an in vitro transcription reaction.

[00165] According to the present invention, 5′ terminal caps may include endogenous caps or cap analogs. According to the present invention, a 5′ terminal cap may comprise a guanine analog. Useful guanine analogs include inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

Viral Sequences

[00166] Additional viral sequences such as, but not limited to, the translation enhancer sequence of the barley yellow dwarf virus (BYDV-PAV) can be engineered and inserted in the 3′ UTR of the polynucleotides, primary constructs or mmRNA of the invention and can stimulate the translation of the construct *in vitro* and *in vivo*. Transfection experiments can be conducted in relevant cell lines at and protein

production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

IRES Sequences

[00167] Further, provided are polynucleotides, primary constructs or mmRNA which may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5′ cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. polynucleotides, primary constructs or mmRNA containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes ("multicistronic nucleic acid molecules"). When polynucleotides, primary constructs or mmRNA are provided with an IRES, further optionally provided is a second translatable region. Examples of IRES sequences that can be used according to the invention include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

Poly-A tails

[00168] During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an mRNA molecules in order to increase stability. Immediately after transcription, the 3' end of the transcript may be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A tail that can be between 100 and 250 residues long.

[00169] It has been discovered that unique poly-A tail lengths provide certain advantages to the polynucleotides, primary constructs or mmRNA of the present invention.

[00170] Generally, the length of a poly-A tail of the present invention is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80,

90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides). In some embodiments, the polynucleotide, primary construct, or mmRNA includes from about 30 to about 3,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to 2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000).

[00171] In one embodiment, the poly-A tail is designed relative to the length of the overall polynucleotides, primary constructs or mmRNA. This design may be based on the length of the coding region, the length of a particular feature or region (such as the first or flanking regions), or based on the length of the ultimate product expressed from the polynucleotides, primary constructs or mmRNA.

[00172] In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the polynucleotides, primary constructs or mmRNA or feature thereof. The poly-A tail may also be designed as a fraction of polynucleotides, primary constructs or mmRNA to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of polynucleotides, primary constructs or mmRNA for Poly-A binding protein may enhance expression.

[00173] Additionally, multiple distinct polynucleotides, primary constructs or mmRNA may be linked together to the PABP (Poly-A binding protein) through the 3′-end using modified nucleotides at the 3′-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

[00174] In one embodiment, the polynucleotide primary constructs of the present invention are designed to include a polyA-G Quartet. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail. The resultant mmRNA construct is assayed for stability, protein production and other parameters including half-life at various time points. It has been discovered that the polyA-G quartet results in protein production equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

Quantification

[00175] In one embodiment, the polynucleotides, primary constructs or mmRNA of the present invention may be quantified in exosomes derived from one or more bodily fluid. As used herein "bodily fluids" include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

[00176] In the quantification method, a sample of not more than 2mL is obtained from the subject and the exosomes isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof. In the analysis, the level or concentration of a polynucleotide, primary construct or mmRNA may be an expression level, presence, absence, truncation or alteration of the administered construct. It is advantageous to correlate the level with one or more clinical phenotypes or with an assay for a human disease biomarker. The assay may be performed using construct specific probes,

cytometry, qRT-PCR, real-time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

[00177] These methods afford the investigator the ability to monitor, in real time, the level of polynucleotides, primary constructs or mmRNA remaining or delivered. This is possible because the polynucleotides, primary constructs or mmRNA of the present invention differ from the endogenous forms due to the structural or chemical modifications.

II. Design and synthesis of mmRNA

[00178] Polynucleotides, primary constructs or mmRNA for use in accordance with the invention may be prepared according to any available technique including, but not limited to chemical synthesis, enzymatic synthesis, which is generally termed *in vitro* transcription (IVT) or enzymatic or chemical cleavage of a longer precursor, etc. Methods of synthesizing RNAs are known in the art (see, *e.g.*, Gait, M.J. (ed.) *Oligonucleotide synthesis: a practical approach*, Oxford [Oxfordshire], Washington, DC: IRL Press, 1984; and Herdewijn, P. (ed.) *Oligonucleotide synthesis: methods and applications*, Methods in Molecular Biology, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

[00179] The process of design and synthesis of the primary constructs of the invention generally includes the steps of gene construction, mRNA production (either with or without modifications) and purification. In the enzymatic synthesis method, a target polynucleotide sequence encoding the polypeptide of interest is first selected for incorporation into a vector which will be amplified to produce a cDNA template. Optionally, the target polynucleotide sequence and/or any flanking sequences may be codon optimized. The cDNA template is then used to produce mRNA through *in vitro* transcription (IVT). After production, the mRNA may undergo purification and clean-up processes. The steps of which are provided in more detail below.

Gene Construction

[00180] The step of gene construction may include, but is not limited to gene synthesis, vector amplification, plasmid purification, plasmid linearization and clean-up, and cDNA template synthesis and clean-up.

Gene Synthesis

[00181] Once a polypeptide of interest, or target, is selected for production, a primary construct is designed. Within the primary construct, a first region of linked nucleosides encoding the polypeptide of interest may be constructed using an open reading frame (ORF) of a selected nucleic acid (DNA or RNA) transcript. The ORF may comprise the wild type ORF, an isoform, variant or a fragment thereof. As used herein, an "open reading frame" or "ORF" is meant to refer to a nucleic acid sequence (DNA or RNA) which is capable of encoding a polypeptide of interest. ORFs often begin with the start codon, ATG and end with a nonsense or termination codon or signal.

[00182] Further, the nucleotide sequence of the first region may be codon optimized. Codon optimization methods are known in the art and may be useful in efforts to achieve one or more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias GC content to increase mRNA stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein trafficking sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites and mRNA degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the mRNA. Codon optimization tools, algorithms and services are known in the art, non-limiting examples include services from GeneArt (Life Technologies) and/or DNA2.0 (Menlo Park CA). In one embodiment, the ORF sequence is optimized using optimization algorithms. Codon options for each amino acid are given in Table 1.

Table 1. Codon Options

Amino Acid	Single Letter Code	Codon Options
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Selenocysteine	Sec	UGA in mRNA in presence of Selenocystein insertion element (SECIS)
Stop codons	Stop	TAA, TAG, TGA

[00183] In one embodiment, at least a portion of the modified mRNA nucleotide sequence may be codon optimized by methods known in the art and/or described herein. After a sequence has been codon optimized it may be further evaluated for regions containing restriction sites. At least one nucleotide within the restriction site regions may be replaced with another nucleotide in order to remove the restriction site from the sequence but the replacement of nucleotides does alter the amino acid sequence which is encoded by the codon optimized nucleotide sequence.

[00184] Features, which may be considered beneficial in some embodiments of the present invention, may be encoded by the primary construct and may flank the ORF as a first or second flanking region. The flanking regions may be incorporated into the primary construct before and/or after optimization of the ORF. It is not required that a primary construct contain both a 5′ and 3′ flanking region. Examples of such features include, but are not limited to, untranslated regions (UTRs), Kozak

sequences, an oligo(dT) sequence, and detectable tags and may include multiple cloning sites which may have XbaI recognition.

[00185] In some embodiments, a 5′ UTR and/or a 3′ UTR may be provided as flanking regions. Multiple 5′ or 3′ UTRs may be included in the flanking regions and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical modifications, before and/or after codon optimization. Combinations of features may be included in the first and second flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail.

[00186] Tables 2 and 3 of co-pending U.S. Provisional Patent Application No 61/737,130 filed December 14, 2012 provide a listing of exemplary UTRs which may be utilized in the primary construct of the present invention as flanking regions. Variants of 5' or 3'UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

[00187] It should be understood that those listed are examples and that any UTR from any gene may be incorporated into the respective first or second flanking region of the primary construct. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present invention to provide artificial UTRs which are not variants of wild type genes. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5′ or 3′ UTR may be inverted, shortened, lengthened, made chimeric with one or more other 5′ UTRs or 3′ UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3′ or 5′ UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of

nucleotides. Any of these changes producing an "altered" UTR (whether 3′ or 5′) comprise a variant UTR.

[00188] In one embodiment, a double, triple or quadruple UTR such as a 5′ or 3′ UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3′ UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[00189] It is also within the scope of the present invention to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[00190] In one embodiment, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature of property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new chimeric primary transcript. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[00191] After optimization (if desired), the primary construct components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes. For example, the optimized construct may be reconstituted and transformed into chemically competent *E. coli,* yeast, neurospora, maize, drosophila, etc. where high copy plasmid-like or chromosome structures occur by methods described herein.

Stop Codons

[00192] In one embodiment, the primary constructs of the present invention may include at least two stop codons before the 3' untranslated region (UTR). The stop

codon may be selected from TGA, TAA and TAG. In one embodiment, the primary constructs of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA.

[00193] In another embodiment, the primary constructs of the present invention may include three stop codons before the 3' untranslated region (UTR).

Vector Amplification

[00194] The vector containing the primary construct is then amplified and the plasmid isolated and purified using methods known in the art such as, but not limited to, a maxi prep using the Invitrogen PURELINK™ HiPure Maxiprep Kit (Carlsbad, CA).

Plasmid Linearization

[00195] The plasmid may then be linearized using methods known in the art such as, but not limited to, the use of restriction enzymes and buffers. The linearization reaction may be purified using methods including, for example Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA), and HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC) and Invitrogen's standard PURELINK™ PCR Kit (Carlsbad, CA). The purification method may be modified depending on the size of the linearization reaction which was conducted. The linearized plasmid is then used to generate cDNA for *in vitro* transcription (IVT) reactions.

cDNA Template Synthesis

[00196] A cDNA template may be synthesized by having a linearized plasmid undergo polymerase chain reaction (PCR). Table 4 of U.S. Provisional Patent Application No 61/737,130 filed December 14, 2012 provides a listing of primers and probes that may be usefully in the PCR reactions of the present invention. It should be understood that the listing is not exhaustive and that primer-probe design for any amplification is within the skill of those in the art. Probes may also contain chemically modified bases to increase base-pairing fidelity to the target molecule and base-pairing strength.

[00197] In one embodiment, the cDNA may be submitted for sequencing analysis before undergoing transcription.

mRNA Production

[00198] The process of mRNA or mmRNA production may include, but is not limited to, *in vitro* transcription, cDNA template removal and RNA clean-up, and mRNA capping and/or tailing reactions.

In Vitro Transcription

[00199] The cDNA produced in the previous step may be transcribed using an *in vitro* transcription (IVT) system. The system typically comprises a transcription buffer, nucleotide triphosphates (NTPs), an RNase inhibitor and a polymerase. The NTPs may be manufactured in house, may be selected from a supplier, or may be synthesized as described herein. The NTPs may be selected from, but are not limited to, those described herein including natural and unnatural (modified) NTPs. The polymerase may be selected from, but is not limited to, T7 RNA polymerase, T3 RNA polymerase and mutant polymerases such as, but not limited to, polymerases able to incorporate modified nucleic acids.

RNA Polymerases

[00200] Any number of RNA polymerases or variants may be used in the design of the primary constructs of the present invention.

[00201] RNA polymerases may be modified by inserting or deleting amino acids of the RNA polymerase sequence. As a non-limiting example, the RNA polymerase may be modified to exhibit an increased ability to incorporate a 2'-modified nucleotide triphosphate compared to an unmodified RNA polymerase (see International Publication WO2008078180 and U.S. Patent 8,101,385; herein incorporated by reference in their entireties).

[00202] Variants may be obtained by evolving an RNA polymerase, optimizing the RNA polymerase amino acid and/or nucleic acid sequence and/or by using other methods known in the art. As a non-limiting example, T7 RNA polymerase variants may be evolved using the continuous directed evolution system set out by Esvelt *et al.* (Nature (2011) 472(7344):499-503; herein incorporated by reference in its entirety) where clones of T7 RNA polymerase may encode at least one mutation such

as, but not limited to, lysine at position 93 substituted for threonine (K93T), I4M, A7T, E63V, V64D, A65E, D66Y, T76N, C125R, S128R, A136T, N165S, G175R, H176L, Y178H, F182L, L196F, G198V, D208Y, E222K, S228A, Q239R, T243N, G259D, M267I, G280C, H300R, D351A, A354S, E356D, L360P, A383V, Y385C, D388Y, S397R, M401T, N410S, K450R, P451T, G452V, E484A, H523L, H524N, G542V, E565K, K577E, K577M, N601S, S684Y, L699I, K713E, N748D, Q754R, E775K, A827V, D851N or L864F. As another non-limiting example, T7 RNA polymerase variants may encode at least mutation as described in U.S. Pub. Nos. 20100120024 and 20070117112; herein incorporated by reference in their entireties. Variants of RNA polymerase may also include, but are not limited to, substitutional variants, conservative amino acid substitution, insertional variants, deletional variants and/or covalent derivatives.

[00203] In one embodiment, the primary construct may be designed to be recognized by the wild type or variant RNA polymerases. In doing so, the primary construct may be modified to contain sites or regions of sequence changes from the wild type or parent primary construct.

[00204] In one embodiment, the primary construct may be designed to include at least one substitution and/or insertion upstream of an RNA polymerase binding or recognition site, downstream of the RNA polymerase binding or recognition site, upstream of the TATA box sequence, downstream of the TATA box sequence of the primary construct but upstream of the coding region of the primary construct, within the 5'UTR, before the 5'UTR and/or after the 5'UTR.

[00205] In one embodiment, the 5'UTR of the primary construct may be replaced by the insertion of at least one region and/or string of nucleotides of the same base. The region and/or string of nucleotides may include, but is not limited to, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 nucleotides and the nucleotides may be natural and/or unnatural. As a non-limiting example, the group of nucleotides may include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

[00206] In one embodiment, the 5'UTR of the primary construct may be replaced by the insertion of at least two regions and/or strings of nucleotides of two different

bases such as, but not limited to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein and/or combinations thereof. For example, the 5'UTR may be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5'UTR may be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

[00207] In one embodiment, the primary construct may include at least one substitution and/or insertion downstream of the transcription start site which may be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion may occur downstream the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site may affect initiation rates, increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Brieba et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety). The modification, substitution and/or insertion of at least one nucleic acid may cause a silent mutation of the nucleic acid sequence or may cause a mutation in the amino acid sequence.

[00208] In one embodiment, the primary construct may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12 or at least 13 guanine bases downstream of the transcription start site.

[00209] In one embodiment, the primary construct may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5 or at least 6 guanine bases in the region just downstream of the transcription start site. As a non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the

guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 thymine, and/or any of the nucleotides described herein.

[00210] In one embodiment, the primary construct may include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The primary construct may include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases may be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4 or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted may be the same base (e.g., all A or all C or all T or all G), two different bases (e.g., A and C, A and T, or C and T), three different bases (e.g., A, C and T or A, C and T) or at least four different bases. As a non-limiting example, the guanine base upstream of the coding region in the primary construct may be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example the substitution of guanine bases in the primary construct may be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt *et al.* Nature (2011) 472(7344):499-503; herein incorporated by reference in its entirety). As a non-limiting example, at least 5 nucleotides may be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides may be the same base type.

cDNA Template Removal and Clean-Up

[00211] The cDNA template may be removed using methods known in the art such as, but not limited to, treatment with Deoxyribonuclease I (DNase I). RNA clean-up may also include a purification method such as, but not limited to, AGENCOURT® CLEANSEQ® system from Beckman Coulter (Danvers, MA), HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC) .

Capping and/or Tailing Reactions

[00212] The primary construct or mmRNA may also undergo capping and/or tailing reactions. A capping reaction may be performed by methods known in the art to add a 5′ cap to the 5′ end of the primary construct. Methods for capping include, but are not limited to, using a Vaccinia Capping enzyme (New England Biolabs, Ipswich, MA).

[00213] A poly-A tailing reaction may be performed by methods known in the art, such as, but not limited to, 2′ O-methyltransferase and by methods as described herein. If the primary construct generated from cDNA does not include a poly-T, it may be beneficial to perform the poly-A-tailing reaction before the primary construct is cleaned.

mRNA Purification

[00214] Primary construct or mmRNA purification may include, but is not limited to, mRNA or mmRNA clean-up, quality assurance and quality control. mRNA or mmRNA clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNA™ oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term "purified" when used in relation to a polynucleotide such as a "purified mRNA or mmRNA" refers to one that is separated from at least one contaminant. As used herein, a "contaminant" is any substance which makes another unfit, impure or inferior. Thus, a purified polynucleotide (e.g., DNA and RNA) is present in a form or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

[00215] A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

[00216] In another embodiment, the mRNA or mmRNA may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

[00217] In one embodiment, the mRNA or mmRNA may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is a NANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified mRNA or mmRNA may be analyzed in order to determine if the mRNA or mmRNA may be of proper size, check that no degradation of the mRNA or mmRNA has occurred. Degradation of the mRNA and/or mmRNA may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Signal Sequences

[00218] The primary constructs or mmRNA may also encode additional features which facilitate trafficking of the polypeptides to therapeutically relevant sites. One such feature which aids in protein trafficking is the signal sequence. As used herein, a "signal sequence" or "signal peptide" is a polynucleotide or polypeptide, respectively, which is from about 9 to 200 nucleotides (3-60 amino acids) in length which is incorporated at the 5′ (or N-terminus) of the coding region or polypeptide encoded, respectively. Addition of these sequences result in trafficking of the encoded polypeptide to the endoplasmic reticulum through one or more secretory pathways. Some signal peptides are cleaved from the protein by signal peptidase after the proteins are transported.

[00219] Signal sequences may be selected from any of those listed in co-pending U.S. Provisional Patent Application No 61/737,130 filed December 14, 2012, the contents of which are incorporated herein by reference. Protein signal sequences which may be incorporated for encoding by the polynucleotides, primary constructs or mmRNA of the invention include signal sequences from α-1-antitrypsin, G-CSF, Factor IX, Prolactin, Albumin, HMMSP38, ornithine carbamoyltransferase, Cytochrome C Oxidase subunit 8A, Type III, bacterial, viral, secretion signals, Vrg-6,

PhoA, OmpA, STI, STII, Amylase, Alpha Factor, Endoglucanase V, Secretion signal, fungal and fibronectin.

[00220] In the table, SS is secretion signal and MLS is mitochondrial leader signal. The primary constructs or mmRNA of the present invention may be designed to encode any of the signal sequences or fragments or variants thereof. These sequences may be included at the beginning of the polypeptide coding region, in the middle or at the terminus or alternatively into a flanking region.

[00221] Additional signal sequences which may be utilized in the present invention include those taught in, for example, databases such as those found at http://www.signalpeptide.de/ or http://proline.bic.nus.edu.sg/spdb/. Those described in US Patents 8,124,379; 7,413,875 and 7,385,034 are also within the scope of the invention and the contents of each are incorporated herein by reference in their entirety.

Target Selection

[00222] According to the present invention, the primary constructs comprise at least a first region of linked nucleosides encoding at least one polypeptide of interest. The polypeptides of interest or "Targets" of the present invention are listed in Table 2 below. Shown in Table 2, in addition to the name and description of the gene encoding the polypeptide of interest are the ENSEMBL Transcript ID (ENST), the ENSEMBL Protein ID (ENSP) and when available the optimized sequence ID (OPT SEQ ID). For any particular gene there may exist one or more variants or isoforms. Where these exist, they are shown in the table as well. It will be appreciated by those of skill in the art that disclosed in the Table are potential flanking regions. These are encoded in each ENST transcript either to the 5′ (upstream) or 3′ (downstream) of the ORF or coding region. The coding region is definitively and specifically disclosed by teaching the ENSP sequence. Consequently, the sequences taught flanking that encoding the protein are considered flanking regions. It is also possible to further characterize the 5′ and 3′ flanking regions by utilizing one or more available databases or algorithms. Databases have annotated the features contained in the flanking regions of the ENST transcripts and these are available in the art.

Table 2. Targets

Target	Gene	Description	ENST	Transcript SEQ ID NO	ENSP	Protein SEQ ID NO
1	LDLR	low density lipoprotein receptor	455727	1	397829	17
2	LDLR	low density lipoprotein receptor	561343	2	454147	18
3	LDLR	low density lipoprotein receptor	558518	3	454071	19
4	LDLR	low density lipoprotein receptor	558013	4	453346	20
5	LDLR	low density lipoprotein receptor	535915	5	440520	21
6	LDLR	low density lipoprotein receptor	545707	6	437639	22
7	LDLR1_D331E PCSK9 mutant	low density lipoprotein receptor/PCSK9 mutant	none	7	none	--
8	LDLR1_L339D PCSK9 mutant	low density lipoprotein receptor/PCSK9 mutant	none	8	none	--
9	LDLR1_N316A PCSK9 Mutant	low density lipoprotein receptor/PCSK9 mutant	none	9	none	--
10	LDLR1_E317A PCSK9 Mutant	low density lipoprotein receptor/PCSK9 mutant	none	10	none	--
11	LDLR1_Y336A PCSK9 Mutant	low density lipoprotein receptor/PCSK9 mutant	none	11	none	--
12	LDLR1_4A PCSK9 mutant	low density lipoprotein receptor/PCSK9 mutant	none	12	none	--
13	CYP7A1	Cholesterol 7alpha hydroxylase	none	13 (combine SEQ ID NO 39, 40 and 41)	none	23
14	PCSK9	proprotein convertase subtilisin/kexin type 9	543384	14	441859	24
15	PCSK9	proprotein convertase subtilisin/kexin type 9	452118	15	401598	25
16	PCSK9	proprotein convertase subtilisin/kexin type 9	302118	16	303208	26

[00223] In one embodiment, the targets of the present invention may be any of the targets described in U.S. Provisional Patent Application No 61/618,862, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Biologics; U.S. Provisional Patent Application No 61/681,645, filed August 10, 2012, entitled

Modified Polynucleotides for the Production of Biologics; U.S. Provisional Patent Application No 61/737,130, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Biologics; U.S. Provisional Patent Application No 61/618,866, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Antibodies; U.S. Provisional Patent Application No 61/681,647, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Antibodies; U.S. Provisional Patent Application No 61/737,134, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Antibodies; U.S. Provisional Patent Application No 61/618,868, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/681,648, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/737,135, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Vaccines; U.S. Provisional Patent Application No 61/618,870, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/681,649, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/737,139, filed December 14, 2012, Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides; U.S. Provisional Patent Application No 61/618,873, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/681,650, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/737,147, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins; U.S. Provisional Patent Application No 61/618,878, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; U.S. Provisional Patent Application No 61/681,654, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; U.S. Provisional Patent Application No 61/737,152, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins; U.S. Provisional Patent Application No 61/618,885, filed April 2, 2012, entitled Modified

Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/681,658, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/737,155, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; U.S. Provisional Patent Application No 61/618,896, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/668,157, filed July 5, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/681,661, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/737,160, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins; U.S. Provisional Patent Application No 61/618,911, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/681,667, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/737,168, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins; U.S. Provisional Patent Application No 61/618,922, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/681,675, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/737,174, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins; U.S. Provisional Patent Application No 61/618,935, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,687, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,184, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/618,945, filed April

2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,696, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,191, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/618,953, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/681,704, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; U.S. Provisional Patent Application No 61/737,203, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, International Application No PCT/US2013/030062, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Biologics and Proteins Associated with Human Disease; International Application No PCT/US2013/030063, filed March 9, 2013, entitled Modified Polynucloetides; International Application No. PCT/US2013/030064, entitled Modified Polynucleotides for the Production of Secreted Proteins; International Application No PCT/US2013/030059, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Membrane Proteins; International Application No. PCT/US2013/030066, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; International Application No. PCT/US2013/030067, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Nuclear Proteins; International Application No. PCT/US2013/030060, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Proteins; International Application No. PCT/US2013/030061, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; International Application No. PCT/US2013/030068, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Cosmetic Proteins and Peptides; International Application No. PCT/US2013/030070, filed March 9, 2013, entitled Modified Polynucleotides for the Production of Oncology-Related Proteins and Peptides; International Application No.

PCT/US2013/031821, filed March 15, 2013, entitled In Vivo Production of Proteins; the contents of each of which are herein incorporated by reference in its entirety.

Protein Cleavage Signals and Sites

[00224] In one embodiment, the polypeptides of the present invention may include at least one protein cleavage signal containing at least one protein cleavage site. The protein cleavage site may be located at the N-terminus, the C-terminus, at any space between the N- and the C- termini such as, but not limited to, half-way between the N- and C-termini, between the N-terminus and the half way point, between the half way point and the C-terminus, and combinations thereof.

[00225] The polypeptides of the present invention may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin or Factor Xa protein cleavage signal. Proprotein convertases are a family of nine proteinases, comprising seven basic amino acid-specific subtilisin-like serine proteinases related to yeast kexin, known as prohormone convertase 1/3 (PC1/3), PC2, furin, PC4, PC5/6, paired basic amino-acid cleaving enzyme 4 (PACE4) and PC7, and two other subtilases that cleave at non-basic residues, called subtilisin kexin isozyme 1 (SKI-1) and proproteinconvertase subtilisin kexin 9 (PCSK9).

[00226] In one embodiment, the primary constructs and mmRNA of the present invention may be engineered such that the primary construct or mmRNA contains at least one encoded protein cleavage signal. The encoded protein cleavage signal may be located before the start codon, after the start codon, before the coding region, within the coding region such as, but not limited to, half way in the coding region, between the start codon and the half way point, between the half way point and the stop codon, after the coding region, before the stop codon, between two stop codons, after the stop codon and combinations thereof.

[00227] In one embodiment, the primary constructs or mmRNA of the present invention may include at least one encoded protein cleavage signal containing at least one protein cleavage site. The encoded protein cleavage signal may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin and/or Factor Xa protein cleavage signal. One of skill in the art may use Table 1 above or other known methods to determine the appropriate encoded protein cleavage signal to

include in the primary constructs or mmRNA of the present invention. For example, starting with a signal sequence and considering the codons of Table 1 one can design a signal for the primary construct which can produce a protein signal in the resulting polypeptide.

[00228] In one embodiment, the polypeptides of the present invention include at least one protein cleavage signal and/or site.

[00229] As a non-limiting example, U.S. Pat. No. 7,374,930 and U.S. Pub. No. 20090227660, herein incorporated by reference in their entireties, use a furin cleavage site to cleave the N-terminal methionine of GLP-1 in the expression product from the Golgi apparatus of the cells. In one embodiment, the polypeptides of the present invention include at least one protein cleavage signal and/or site with the proviso that the polypeptide is not GLP-1.

[00230] In one embodiment, the primary constructs or mmRNA of the present invention includes at least one encoded protein cleavage signal and/or site.

[00231] In one embodiment, the primary constructs or mmRNA of the present invention includes at least one encoded protein cleavage signal and/or site with the proviso that the primary construct or mmRNA does not encode GLP-1.

[00232] In one embodiment, theprimary constructs or mmRNA of the present invention may include more than one coding region. Where multiple coding regions are present in the primary construct or mmRNA of the present invention, the multiple coding regions may be separated by encoded protein cleavage sites. As a non-limiting example, the primary construct or mmRNA may be signed in an ordered pattern. On such pattern follows AXBY form where A and B are coding regions which may be the same or different coding regions and/or may encode the same or different polypeptides, and X and Y are encoded protein cleavage signals which may encode the same or different protein cleavage signals. A second such pattern follows the form AXYBZ where A and B are coding regions which may be the same or different coding regions and/or may encode the same or different polypeptides, and X, Y and Z are encoded protein cleavage signals which may encode the same or different protein cleavage signals. A third pattern follows the form ABXCY where A, B and C are coding regions which may be the same or different coding regions

and/or may encode the same or different polypeptides, and X and Y are encoded protein cleavage signals which may encode the same or different protein cleavage signals.

[00233] In one embodiment, the polypeptides, primary constructs and mmRNA can also contain sequences that encode protein cleavage sites so that the polypeptides, primary constructs and mmRNA can be released from a carrier region or a fusion partner by treatment with a specific protease for said protein cleavage site.

III. Modifications

[00234] Herein, in a polynucleotide (such as a primary construct or an mRNA molecule), the terms "modification" or, as appropriate, "modified" refer to modification with respect to A, G, U or C ribonucleotides. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties. In a polypeptide, the term "modification" refers to a modification as compared to the canonical set of 20 amino acids, moiety)

[00235] The modifications may be various distinct modifications. In some embodiments, the coding region, the flanking regions and/or the terminal regions may contain one, two, or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified polynucleotide, primary construct, or mmRNA introduced to a cell may exhibit reduced degradation in the cell, as compared to an unmodified polynucleotide, primary construct, or mmRNA.

[00236] The polynucleotides, primary constructs, and mmRNA can include any useful modification, such as to the sugar, the nucleobase, or the internucleoside linkage (*e.g.* to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage. Modifications according to the present invention may be modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids

(GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[00237] As described herein, the polynucleotides, primary constructs, and mmRNA of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced. Featues of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc, and/or 3) termination or reduction in protein translation.

[00238] In certain embodiments, it may desirable to intracellularly degrade a modified nucleic acid molecule introduced into the cell. For example, degradation of a modified nucleic acid molecule may be preferable if precise timing of protein production is desired. Thus, in some embodiments, the invention provides a modified nucleic acid molecule containing a degradation domain, which is capable of being acted on in a directed manner within a cell.

[00239] The polynucleotides, primary constructs, and mmRNA can optionally include other agents (e.g., RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers, vectors, etc.). In some embodiments, the polynucleotides, primary constructs, or mmRNA may include one or more messenger RNAs (mRNAs) and one or more modified nucleoside or nucleotides (e.g., mmRNA molecules). Details for these polynucleotides, primary constructs, and mmRNA follow.

Polynucleotides and Primary Constructs

[00240] The polynucleotides, primary constructs, and mmRNA of the invention includes a first region of linked nucleosides encoding a polypeptide of interest, a first flanking region located at the 5′ terminus of the first region, and a second flanking region located at the 3′ terminus of the first region.

[00241] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having any base, sugar, backbone, building block or other structure or formula, including but not limited to those of Formulas I through IX or any substructures thereof as described in International Application

PCT/US12/58519 filed October 3, 2012 (Attorney Docket Number: M009.20), the contents of which are incorporated herein by reference in their entirety. Such structures include modifications to the sugar, nucleobase, internucleoside linkage, or combinations thereof.

[00242] Combinations of chemical modifications include those taught in including but not limited to those described in International Application PCT/US12/58519 filed October 3, 2012 (Attorney Docket Number: M009.20), the contents of which are incorporated herein by reference in their entirety.

[00243] The synthesis of polynucleotides, primary constructs or mmRNA of the present invention may be according to the methods described in International Application PCT/US12/58519 filed October 3, 2012 (Attorney Docket Number: M009.20), the contents of which are incorporated herein by reference in their entirety.

[00244] In some embodiments, the nucleobase selected from the group consisting of cytosine, guanine, adenine, and uracil.

[00245] In some embodiments, the modified nucleobase is a modified uracil. Exemplary nucleobases and nucleosides having a modified uracil include pseudouridine (ψ), pyridin-4-one ribonucleoside, 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine (s^2U), 4-thio-uridine (s^4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (ho^5U), 5-aminoallyl-uridine, 5-halo-uridine (e.g., 5-iodo-uridine or 5-bromo-uridine), 3-methyl-uridine (m^3U), 5-methoxy-uridine (mo^5U), uridine 5-oxyacetic acid (cmo^5U), uridine 5-oxyacetic acid methyl ester ($mcmo^5U$), 5-carboxymethyl-uridine (cm^5U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine (chm^5U), 5-carboxyhydroxymethyl-uridine methyl ester ($mchm^5U$), 5-methoxycarbonylmethyl-uridine (mcm^5U), 5-methoxycarbonylmethyl-2-thio-uridine (mcm^5s^2U), 5-aminomethyl-2-thio-uridine (nm^5s^2U), 5-methylaminomethyl-uridine (mnm^5U), 5-methylaminomethyl-2-thio-uridine (mnm^5s^2U), 5-methylaminomethyl-2-seleno-uridine (mnm^5se^2U), 5-carbamoylmethyl-uridine (ncm^5U), 5-carboxymethylaminomethyl-uridine ($cmnm^5U$), 5-carboxymethylaminomethyl-2-thio-uridine ($cmnm^5s^2U$), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyl-uridine (τm^5U), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine(τm^5s^2U), 1-taurinomethyl-4-thio-

pseudouridine, 5-methyl-uridine (m^5U, i.e., having the nucleobase deoxythymine), 1-methyl-pseudouridine ($m^1\psi$), 5-methyl-2-thio-uridine (m^5s^2U), 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine (m^5D), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uridine (acp^3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\ \psi$), 5-(isopentenylaminomethyl)uridine (inm^5U), 5-(isopentenylaminomethyl)-2-thio-uridine (inm^5s^2U), α-thio-uridine, 2′-O-methyl-uridine (Um), 5,2′-O-dimethyl-uridine (m^5Um), 2′-O-methyl-pseudouridine (ψm), 2-thio-2′-O-methyl-uridine (s^2Um), 5-methoxycarbonylmethyl-2′-O-methyl-uridine (mcm^5Um), 5-carbamoylmethyl-2′-O-methyl-uridine (ncm^5Um), 5-carboxymethylaminomethyl-2′-O-methyl-uridine ($cmnm^5Um$), 3,2′-O-dimethyl-uridine (m^3Um), 5-(isopentenylaminomethyl)-2′-O-methyl-uridine (inm^5Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, and 5-[3-(1-E-propenylamino)uridine.

[00246] In some embodiments, the modified nucleobase is a modified cytosine. Exemplary nucleobases and nucleosides having a modified cytosine include 5-aza-cytidine, 6-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine (m^3C), N4-acetyl-cytidine (ac^4C), 5-formyl-cytidine (f^5C), N4-methyl-cytidine (m^4C), 5-methyl-cytidine (m^5C), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm^5C), 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine (s^2C), 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k_2C), α-thio-cytidine, 2′-O-methyl-cytidine (Cm), 5,2′-O-dimethyl-cytidine (m^5Cm),

N4-acetyl-2′-O-methyl-cytidine (ac^4Cm), N4,2′-O-dimethyl-cytidine (m^4Cm), 5-formyl-2′-O-methyl-cytidine (f^5Cm), N4,N4,2′-O-trimethyl-cytidine ($m^4{}_2Cm$), 1-thio-cytidine, 2'-F-ara-cytidine, 2'-F-cytidine, and 2'-OH-ara-cytidine.

[00247] In some embodiments, the modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified adenine include 2-amino-purine, 2, 6-diaminopurine, 2-amino-6-halo-purine (e.g., 2-amino-6-chloro-purine), 6-halo-purine (e.g., 6-chloro-purine), 2-amino-6-methyl-purine, 8-azido-adenosine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-amino-purine, 7-deaza-8-aza-2-amino-purine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyl-adenosine (m^1A), 2-methyl-adenine (m^2A), N6-methyl-adenosine (m^6A), 2-methylthio-N6-methyl-adenosine (ms^2m^6A), N6-isopentenyl-adenosine (i^6A), 2-methylthio-N6-isopentenyl-adenosine (ms^2i^6A), N6-(cis-hydroxyisopentenyl)adenosine (io^6A), 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine (ms^2io^6A), N6-glycinylcarbamoyl-adenosine (g^6A), N6-threonylcarbamoyl-adenosine (t^6A), N6-methyl-N6-threonylcarbamoyl-adenosine (m^6t^6A), 2-methylthio-N6-threonylcarbamoyl-adenosine (ms^2g^6A), N6,N6-dimethyl-adenosine ($m^6{}_2A$), N6-hydroxynorvalylcarbamoyl-adenosine (hn^6A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenosine (ms^2hn^6A), N6-acetyl-adenosine (ac^6A), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, α-thio-adenosine, 2′-O-methyl-adenosine (Am), N6,2′-O-dimethyl-adenosine (m^6Am), N6,N6,2′-O-trimethyl-adenosine ($m^6{}_2Am$), 1,2′-O-dimethyl-adenosine (m^1Am), 2′-O-ribosyladenosine (phosphate) (Ar(p)), 2-amino-N6-methyl-purine, 1-thio-adenosine, 8-azido-adenosine, 2'-F-ara-adenosine, 2'-F-adenosine, 2'-OH-ara-adenosine, and N6-(19-amino-pentaoxanonadecyl)-adenosine.

[00248] In some embodiments, the modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified guanine include inosine (I), 1-methyl-inosine (m^1I), wyosine (imG), methylwyosine (mimG), 4-demethyl-wyosine (imG-14), isowyosine (imG2), wybutosine (yW), peroxywybutosine (o_2yW), hydroxywybutosine (OHyW), undermodified hydroxywybutosine (OHyW*), 7-deaza-guanosine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ),

mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanosine ($preQ_0$), 7-aminomethyl-7-deaza-guanosine ($preQ_1$), archaeosine (G^+), 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine (m^7G), 6-thio-7-methyl-guanosine, 7-methyl-inosine, 6-methoxy-guanosine, 1-methyl-guanosine (m^1G), N2-methyl-guanosine (m^2G), N2,N2-dimethyl-guanosine ($m^2{}_2G$), N2,7-dimethyl-guanosine ($m^{2,7}G$), N2, N2,7-dimethyl-guanosine ($m^{2,2,7}G$), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, N2,N2-dimethyl-6-thio-guanosine, α-thio-guanosine, 2′-O-methyl-guanosine (Gm), N2-methyl-2′-O-methyl-guanosine (m^2Gm), N2,N2-dimethyl-2′-O-methyl-guanosine ($m^2{}_2Gm$), 1-methyl-2′-O-methyl-guanosine (m^1Gm), N2,7-dimethyl-2′-O-methyl-guanosine ($m^{2,7}Gm$), 2′-O-methyl-inosine (Im), 1,2′-O-dimethyl-inosine (m^1Im), and 2′-O-ribosylguanosine (phosphate) (Gr(p)).

[00249] The nucleobase of the nucleotide can be independently selected from a purine, a pyrimidine, a purine or pyrimidine analog. For example, the nucleobase can each be independently selected from adenine, cytosine, guanine, uracil, or hypoxanthine. In another embodiment, the nucleobase can also include, for example, naturally-occurring and synthetic derivatives of a base, including pyrazolo[3,4-d]pyrimidines, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo (*e.g.,* 8-bromo), 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, deazaguanine, 7-deazaguanine, 3-deazaguanine, deazaadenine, 7-deazaadenine, 3-deazaadenine, pyrazolo[3,4-d]pyrimidine, imidazo[1,5-a]1,3,5 triazinones, 9-deazapurines, imidazo[4,5-d]pyrazines, thiazolo[4,5-d]pyrimidines, pyrazin-2-ones, 1,2,4-triazine, pyridazine; and 1,3,5 triazine. When the nucleotides are depicted using the shorthand A, G, C, T or U, each letter refers to the representative base and/or

derivatives thereof, *e.g.*, A includes adenine or adenine analogs, *e.g.,* 7-deaza adenine).

[00250] Modified nucleosides and nucleotides (e.g., building block molecules) can be prepared according to the synthetic methods described in Ogata et al., J. Org. Chem. 74:2585-2588 (2009); Purmal et al., Nucl. Acids Res. 22(1): 72-78, (1994); Fukuhara et al., Biochemistry, 1(4): 563-568 (1962); and Xu et al., Tetrahedron, 48(9): 1729-1740 (1992), each of which are incorporated by reference in their entirety.

[00251] The polypeptides, primary constructs, and mmRNA of the invention may or may not be uniformly modified along the entire length of the molecule. For example, one or more or all types of nucleotide (*e.g.*, purine or pyrimidine, or any one or more or all of A, G, U, C) may or may not be uniformly modified in a polynucleotide of the invention, or in a given predetermined sequence region thereof (*e.g.* one or more of the sequence regions represented in Figure 1). In some embodiments, all nucleotides X in a polynucleotide of the invention (or in a given sequence region thereof) are modified, wherein X may any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

[00252] Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in the polynucleotide, primary construct, or mmRNA. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a polynucleotide, primary construct, or mmRNA such that the function of the polynucleotide, primary construct, or mmRNA is not substantially decreased. A modification may also be a 5′ or 3′ terminal modification. The polynucleotide, primary construct, or mmRNA may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e. any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%,

from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%).

[00253] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a modified pyrimidine (e.g., a modified uracil/uridine/U or modified cytosine/cytidine/C). In some embodiments, the uracil or uridine (generally: U) in the polynucleotide, primary construct, or mmRNA molecule may be replaced with from about 1% to about 100% of a modified uracil or modified uridine (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100% of a modified uracil or modified uridine). The modified uracil or uridine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein).

[00254] In some embodiments, the cytosine or cytidine (generally: C) in the polynucleotide, primary construct, or mmRNA molecule may be replaced with from about 1% to about 100% of a modified cytosine or modified cytidine (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from

10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100% of a modified cytosine or modified cytidine). The modified cytosine or cytidine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein).

[00255] In some embodiments, the polynucleotide, primary construct, or mmRNA is translatable.

[00256] Other components of polynucleotides, primary constructs, and mmRNA are optional, and are beneficial in some embodiments. For example, a 5′ untranslated region (UTR) and/or a 3′UTR are provided, wherein either or both may independently contain one or more different nucleotide modifications. In such embodiments, nucleotide modifications may also be present in the translatable region. Also provided are polynucleotides, primary constructs, and mmRNA containing a Kozak sequence.

[00257] In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

[00258] In some embodiments, at least 25% of the uracils are replaced by a compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

[00259] In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14), and at least 25% of the uracils are replaced by a

compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

IV. Pharmaceutical Compositions

Formulation, Administration, Delivery and Dosing

[00260] The present invention provides polynucleotides, primary constructs and mmRNA compositions and complexes in combination with one or more pharmaceutically acceptable excipients. Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g. therapeutically and/or prophylactically active substances. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in *Remington: The Science and Practice of Pharmacy* 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety).

[00261] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to polynucleotides, primary constructs and mmRNA to be delivered as described herein.

[00262] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, *e.g.,* to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats;

and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[00263] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[00264] A pharmaceutical composition in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[00265] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

[00266] Any of the polynucleotides, primary constructs and mmRNA described herein may be formulated as described in International Application No PCT/US2012/069610, filed December 14, 2012, entitled Modified Nucleoside, Nucleotide, and Nucleic Acid Compositions, the contents of which is herein incorporated by reference in its entirety.

Formulations

[00267] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot

formulation of the polynucleotide, primary construct, or mmRNA); (4) alter the biodistribution (e.g., target the polynucleotide, primary construct, or mmRNA to specific tissues or cell types); (5) increase the translation of encoded protein *in vivo*; and/or (6) alter the release profile of encoded protein *in vivo*. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients of the present invention can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with polynucleotide, primary construct, or mmRNA (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. Accordingly, the formulations of the invention can include one or more excipients, each in an amount that together increases the stability of the polynucleotide, primary construct, or mmRNA, increases cell transfection by the polynucleotide, primary construct, or mmRNA, increases the expression of polynucleotide, primary construct, or mmRNA encoded protein, and/or alters the release profile of polynucleotide, primary construct, or mmRNA encoded proteins. Further, the primary construct and mmRNA of the present invention may be formulated using self-assembled nucleic acid nanoparticles.

[00268] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[00269] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient may generally be equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage including, but not limited to, one-half or one-third of such a dosage.

[00270] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient.

[00271] In some embodiments, the formulations described herein may contain at least one mmRNA. As a non-limiting example, the formulations may contain 1, 2, 3, 4 or 5 mmRNA. In one embodiment the formulation may contain modified mRNA encoding proteins selected from categories such as, but not limited to, human proteins, veterinary proteins, bacterial proteins, biological proteins, antibodies, immunogenic proteins, therapeutic peptides and proteins, secreted proteins, plasma membrane proteins, cytoplasmic and cytoskeletal proteins, intrancellular membrane bound proteins, nuclear proteins, proteins associated with human disease and/or proteins associated with non-human diseases. In one embodiment, the formulation contains at least three modified mRNA encoding proteins. In one embodiment, the formulation contains at least five modified mRNA encoding proteins.

[00272] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[00273] In some embodiments, the particle size of the lipid nanoparticle may be increased and/or decreased. The change in particle size may be able to help counter biological reaction such as, but not limited to, inflammation or may increase the biological effect of the modified mRNA delivered to mammals.

[00274] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, surface active agents and/or emulsifiers, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the pharmaceutical formulations of the invention.

[00275] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be administered in or with, formulated in or delivered with nanostructures that can sequester molecules such as cholesterol. Non-limiting examples of these nanostructures and methods of making these nanostructures are described in US Patent Publication No. US20130195759, the contents of which are herein incorporated by reference in its entirety. Exemplary structures of these nanostructures are shown in Figure 1 of US Patent Publication No. US20130195759, the contents of which are herein incorporated by reference in its entirety, and may include a core and a shell surrounding the core.

Lipidoids

[00276] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of polynucleotides, primary constructs or mmRNA (see Mahon et al., Bioconjug Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al., Nat Biotechnol. 2008 26:561-569; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-3001; all of which are incorporated herein in their entireties).

[00277] While these lipidoids have been used to effectively deliver double stranded small interfering RNA molecules in rodents and non-human primates (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Frank-Kamenetsky et al., Proc Natl Acad Sci U S A. 2008 105:11915-11920; Akinc et al., Mol Ther. 2009 17:872-879; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Leuschner et al., Nat Biotechnol.

2011 29:1005-1010; all of which is incorporated herein in their entirety), the present disclosure describes their formulation and use in delivering single stranded polynucleotides, primary constructs, or mmRNA. Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective delivery of the polynucleotide, primary construct, or mmRNA, as judged by the production of an encoded protein, following the injection of a lipidoid formulation via localized and/or systemic routes of administration. Lipidoid complexes of polynucleotides, primary constructs, or mmRNA can be administered by various means including, but not limited to, intravenous, intramuscular, or subcutaneous routes.

[00278] *In vivo* delivery of nucleic acids may be affected by many parameters, including, but not limited to, the formulation composition, nature of particle PEGylation, degree of loading, oligonucleotide to lipid ratio, and biophysical parameters such as particle size (Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). As an example, small changes in the anchor chain length of poly(ethylene glycol) (PEG) lipids may result in significant effects on in vivo efficacy. Formulations with the different lipidoids, including, but not limited to penta[3-(1-laurylaminopropionyl)]-triethylenetetramine hydrochloride (TETA–5LAP; aka 98N12-5, see Murugaiah et al., Analytical Biochemistry, 401:61 (2010)), C12-200 (including derivatives and variants), and MD1, can be tested for *in vivo* activity.

[00279] The lipidoid referred to herein as "98N12-5" is disclosed by Akinc et al., Mol Ther. 2009 17:872-879 and is incorporated by reference in its entirety.

[00280] The lipidoid referred to herein as "C12-200" is disclosed by Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 and Liu and Huang, Molecular Therapy. 2010 669-670; both of which are herein incorporated by reference in their entirety. The lipidoid formulations can include particles comprising either 3 or 4 or more components in addition to polynucleotide, primary construct, or mmRNA. As an example, formulations with certain lipidoids, include, but are not limited to, 98N12-5 and may contain 42% lipidoid, 48% cholesterol and 10% PEG (C14 alkyl chain length). As another example, formulations with certain lipidoids, include, but

are not limited to, C12-200 and may contain 50% lipidoid, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and 1.5% PEG-DMG.

[00281] In one embodiment, a polynucleotide, primary construct, or mmRNA formulated with a lipidoid for systemic intravenous administration can target the liver. For example, a final optimized intravenous formulation using polynucleotide, primary construct, or mmRNA, and comprising a lipid molar composition of 42% 98N12-5, 48% cholesterol, and 10% PEG-lipid with a final weight ratio of about 7.5 to 1 total lipid to polynucleotide, primary construct, or mmRNA, and a C14 alkyl chain length on the PEG lipid, with a mean particle size of roughly 50–60 nm, can result in the distribution of the formulation to be greater than 90% to the liver (see, Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated in its entirety). In another example, an intravenous formulation using a C12-200 (see US provisional application 61/175,770 and published international application WO2010129709, each of which is herein incorporated by reference in their entirety) lipidoid may have a molar ratio of 50/10/38.5/1.5 of C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG, with a weight ratio of 7 to 1 total lipid to polynucleotide, primary construct, or mmRNA, and a mean particle size of 80 nm may be effective to deliver polynucleotide, primary construct, or mmRNA to hepatocytes (see, Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 herein incorporated by reference). In another embodiment, an MD1 lipidoid-containing formulation may be used to effectively deliver polynucleotide, primary construct, or mmRNA to hepatocytes *in vivo*. The characteristics of optimized lipidoid formulations for intramuscular or subcutaneous routes may vary significantly depending on the target cell type and the ability of formulations to diffuse through the extracellular matrix into the blood stream. While a particle size of less than 150 nm may be desired for effective hepatocyte delivery due to the size of the endothelial fenestrae (see, Akinc et al., Mol Ther. 2009 17:872-879 herein incorporated by reference), use of a lipidoid-formulated polynucleotide, primary construct, or mmRNA to deliver the formulation to other cells types including, but not limited to, endothelial cells, myeloid cells, and muscle cells may not be similarly size-limited. Use of lipidoid formulations to deliver siRNA in vivo to other non-hepatocyte cells

such as myeloid cells and endothelium has been reported (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; Cho et al. Adv. Funct. Mater. 2009 19:3112-3118; 8th International Judah Folkman Conference, Cambridge, MA October 8-9, 2010 herein incorporated by reference in its entirety). Effective delivery to myeloid cells, such as monocytes, lipidoid formulations may have a similar component molar ratio. Different ratios of lipidoids and other components including, but not limited to, disteroylphosphatidyl choline, cholesterol and PEG-DMG, may be used to optimize the formulation of the polynucleotide, primary construct, or mmRNA for delivery to different cell types including, but not limited to, hepatocytes, myeloid cells, muscle cells, etc. For example, the component molar ratio may include, but is not limited to, 50% C12-200, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and %1.5 PEG-DMG (see Leuschner et al., Nat Biotechnol 2011 29:1005-1010; herein incorporated by reference in its entirety). The use of lipidoid formulations for the localized delivery of nucleic acids to cells (such as, but not limited to, adipose cells and muscle cells) via either subcutaneous or intramuscular delivery, may not require all of the formulation components desired for systemic delivery, and as such may comprise only the lipidoid and the polynucleotide, primary construct, or mmRNA.

[00282] Combinations of different lipidoids may be used to improve the efficacy of polynucleotide, primary construct, or mmRNA directed protein production as the lipidoids may be able to increase cell transfection by the polynucleotide, primary construct, or mmRNA; and/or increase the translation of encoded protein (see Whitehead et al., Mol. Ther. 2011, 19:1688-1694, herein incorporated by reference in its entirety).

Liposomes, Lipoplexes, and Lipid Nanoparticles

[00283] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of polynucleotide, primary construct, or mmRNA include liposomes. Liposomes are artificially-prepared vesicles which may primarily be composed of a lipid bilayer and may be used as a delivery vehicle for the administration of nutrients and pharmaceutical formulations. Liposomes can be of

different sizes such as, but not limited to, a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, a small unicellular vesicle (SUV) which may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) which may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations.

[00284] The formation of liposomes may depend on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients , the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

[00285] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; herein incorporated by reference in its entirety) and liposomes which may deliver small molecule drugs such as, but not limited to, DOXIL® from Janssen Biotech, Inc. (Horsham, PA).

[00286] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery in vitro and in vivo (see Wheeler et al. Gene Therapy. 1999 6:271-281; Zhang et al. Gene Therapy. 1999 6:1438-1447; Jeffs et al. Pharm Res.

2005 22:362-372; Morrissey et al., Nat Biotechnol. 2005 2:1002-1007; Zimmermann et al., Nature. 2006 441:111-114; Heyes et al. J Contr Rel. 2005 107:276-287; Semple et al. Nature Biotech. 2010 28:172-176; Judge et al. J Clin Invest. 2009 119:661-673; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein in their entireties). The original manufacture method by Wheeler et al. was a detergent dialysis method, which was later improved by Jeffs et al. and is referred to as the spontaneous vesicle formation method. The liposome formulations are composed of 3 to 4 lipid components in addition to the polynucleotide, primary construct, or mmRNA. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODMA), as described by Jeffs et al. As another example, certain liposome formulations may contain, but are not limited to, 48% cholesterol, 20% DSPC, 2% PEG-c-DMA, and 30% cationic lipid, where the cationic lipid can be 1,2-distearloxy-*N,N*-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or 1,2-dilinolenyloxy-3-dimethylaminopropane (DLenDMA), as described by Heyes et al.

[00287] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers.

[00288] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Pub. No. 20120178702, herein incorporated by reference in its entirety. As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine. In another embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid-polycation complex which may further include a neutral lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

[00289] The liposome formulation may be influenced by, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the

nature of the PEGylation, ratio of all components and biophysical parameters such as size. In one example by Semple et al. (Semple et al. Nature Biotech. 2010 28:172-176), the liposome formulation was composed of 57.1 % cationic lipid, 7.1% dipalmitoylphosphatidylcholine, 34.3 % cholesterol, and 1.4% PEG-c-DMA. As another example, changing the composition of the cationic lipid could more effectively deliver siRNA to various antigen presenting cells (Basha et al. Mol Ther. 2011 19:2186-2200; herein incorporated by reference in its entirety).

[00290] In some embodiments, the ratio of PEG in the LNP formulations may be increased or decreased and/or the carbon chain length of the PEG lipid may be modified from C14 to C18 to alter the pharmacokinetics and/or biodistribution of the LNP formulations. As a non-limiting example, LNP formulations may contain 1-5% of the lipid molar ratio of PEG-c-DOMG as compared to the cationic lipid, DSPC and cholesterol. In another embodiment the PEG-c-DOMG may be replaced with a PEG lipid such as, but not limited to, PEG- DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene glycol) or PEG-DPG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol). The cationic lipid may be selected from any lipid known in the art such as, but not limited to, DLin-MC3-DMA, DLin-DMA, C12-200 and DLin-KC2-DMA.

[00291] In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865 and WO2008103276, US Patent Nos. 7,893,302 and 7,404,969 and US Patent Publication No. US20100036115; each of which is herein incorporated by reference in their entirety. In another embodiment, the cationic lipid may be selected from, but not limited to, formula A described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365 and WO2012044638; each of which is herein incorporated by reference in their entirety. In yet another embodiment, the cationic lipid may be selected from, but not limited to, formula CLI-CLXXIX of International Publication

No. WO2008103276, formula CLI-CLXXIX of US Patent No. 7,893,302, formula CLI-CLXXXXII of US Patent No. 7,404,969 and formula I-VI of US Patent Publication No. US20100036115; each of which is herein incorporated by reference in their entirety. As a non-limiting example, the cationic lipid may be selected from (20Z,23Z)-N,N-dimethylnonacosa-20,23-dien-10-amine, (17Z,20Z)-N,N-dimemylhexacosa-17,20-dien-9-amine, (1 Z, 19Z)-N5N~dimethylpentacosa~l 6, 19-dien-8-amine, (13Z,16Z)-N,N-dimethyldocosa-13J16-dien-5-amine, (12Z,15Z)-NJN-dimethylhenicosa-12,15-dien-4-amine, (14Z,17Z)-N,N-dimethyltricosa-14,17-dien-6-amine, (15Z,18Z)-N,N-dimethyltetracosa-15,18-dien-7-amine, (18Z,21Z)-N,N-dimethylheptacosa-18,21-dien-10-amine, (15Z,18Z)-N,N-dimethyltetracosa-15,18-dien-5-amine, (14Z,17Z)-N,N-dimethyltricosa-14,17-dien-4-amine, (19Z,22Z)-N,N-dimeihyloctacosa-19,22-dien-9-amine, (18Z,21 Z)-N ,N-dimethylheptacosa- 18 ,21 -dien-8 –amine, (17Z,20Z)-N,N-dimethylhexacosa- 17,,20-dien-7-amine, (16Z;19Z)-N,N-dimethylpentacosa-16,19-dien-6-amine, (22Z,25Z)-N,N-dimethylhentriaconta-22,25-dien- 10-amine, (21 Z ,24Z)-N;N-dimethyltriaconta-21 ,24-dien-9-amine, (18Z)-N,N-dimetylheptacos-18-en-10-amine, (17Z)-N,N-dimethylhexacos-17-en-9-amine, (19Z,22Z)-NJN-dimethyloctacosa-19,22-dien-7-amine, N,N-dimethylheptacosan-10-amine, (20Z,23Z)-N-ethyl-N-methylnonacosa-20J23-dien-l 0-amine, 1-[(1 lZ,14Z)-l-nonylicosa-l 1,14-dien-l-yl] pyrrolidine, (20Z)-N,N-dimethylheptacos-20-en-l 0-amine, (15Z)-N,N-dimethyl eptacos-15-en-l 0-amine, (14Z)-N,N-dimethylnonacos-14-en-l 0-amine, (17Z)-N,N-dimethylnonacos-17-en-l 0-amine, (24Z)-N,N-dimethyltritriacont-24-en-l 0-amine, (20Z)-N,N-dimethylnonacos-20-en-l 0-amine, (22Z)-N,N-dimethylhentriacont-22-en-l 0-amine, (16Z)-N,N-dimethylpentacos-16-en-8-amine, (12Z, 15Z)-N,N-dimethyl-2-nonylhenico sa- 12 , 15 -dien- 1 –amine, (13Z, 16Z)-N,N-dimethyl-3-nonyldocosa-l 3, 16-dien-l –amine, N,N-dimethyl-l-[(l S,2R)-2-octylcyclopropyl] eptadecan-8-amine, 1 -[(1 S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan- 10-amine, N,N-dimethyl- 1 - [(1 S ,2R)-2-octylcyclopropyl]nonadecan- 10-amine, N,N-dimethyl-21~[(lS,2R)-2-octylcyclopropyl]henicosan-l 0-amine, N,N-dimethyl- 1 -[(1 S ,2S)-2- { [(lR,2R)-2-pentylcycIopropyl]methyl} cyclopropyl]nonadecan- 10-amine, N,N-dimethyl- 1 -[(1 S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyH -[(lR,2S)-2-

undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3- {7-[(1 S,2R)-2-octylcyclopropyl]heptyl} dodecan- 1 –amine, 1 - [(1 R,2 S)-2-hepty lcyclopropy 1] - N,N-dimethyloctadecan-9 –amine, 1-[(1 S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-l-[(lS,2R)-2-octylcyclopropyl]pentadecan-8-amine, R -N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S -N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy) methyl]ethyl}pyrrolidine, (2S)-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct -5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy) methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(nonyloxy)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine (Compound 9); (2S)-N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethy1-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-l3,16-dien-l-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)-N,N-dimethyl-H(1-metoyloctyl)oxy]-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(octyloxy)-3-({8-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]octyl}oxy)propan-2-amine, N,N-dimethyl-1-{[8-(2-oc1ylcyclopropyl)octyl]oxy}-3-(octyloxy)propan-2-amine and (11E,20Z,23Z)-N;N-dimethylnonacosa-l1,20,2-trien-10-amine or a pharmaceutically acceptable salt or stereoisomer thereof.

[00292] In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724 and WO201021865; each of which is herein incorporated by reference in their entirety.

[00293] In one embodiment, the LNP formulations of the polynucleotides, primary constructs and/or mmRNA may contain PEG-c-DOMG 3% lipid molar ratio. In another embodiment, the LNP formulations of the polynucleotides, primary constructs and/or mmRNA may contain PEG-c-DOMG 1.5% lipid molar ratio.

[00294] In one embodiment, the pharmaceutical compositions of the polynucleotides, primary constructs and/or mmRNA may include at least one of the PEGylated lipids described in International Publication No. 2012099755, herein incorporated by reference.

[00295] In one embodiment, the LNP formulation may contain PEG-DMG 2000 (1,2-dimyristoyl-sn-glycero-3-phophoethanolamine-N-[methoxy(polyethylene glycol)-2000). In one embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art and at least one other component. In another embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art, DSPC and cholesterol. As a non-limiting example, the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol. As another non-limiting example the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol in a molar ratio of 2:40:10:48 (see Geall et al., Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294).

[00296] In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO2011127255 or WO2008103276, each of which is herein incorporated by reference in their entirety. As a non-limiting example, modified RNA described herein may be encapsulated in LNP formulations as described in WO2011127255 and/or WO2008103276; each of which is herein incorporated by reference in their entirety.

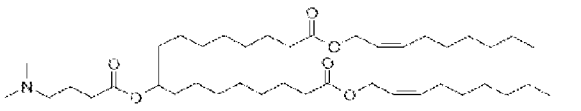
[00297] In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US20050222064; herein incorporated by reference in its entirety. In another embodiment, the LNP formulations comprising a polycationic composition may be used for the delivery of the modified RNA described herein *in vivo* and/or *in vitro*.

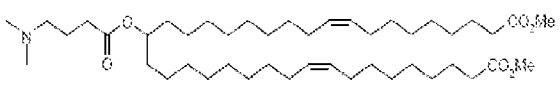
[00298] In one embodiment, the LNP formulations described herein may additionally comprise a permeability enhancer molecule. Non-limiting permeability enhancer molecules are described in US Patent Publication No. US20050222064; herein incorporated by reference in its entirety.

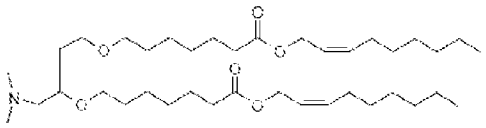
[00299] In one embodiment, the pharmaceutical compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713)) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

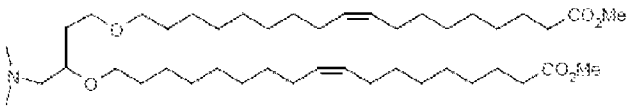
[00300] Lipid nanoparticle formulations may be improved by replacing the cationic lipid with a biodegradable cationic lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Ionizable cationic lipids, such as, but not limited to, DLinDMA, DLin-KC2-DMA, and DLin-MC3-DMA, have been shown to accumulate in plasma and tissues over time and may be a potential source of toxicity. The rapid metabolism of the rapidly eliminated lipids can improve the tolerability and therapeutic index of the lipid nanoparticles by an order of magnitude from a 1 mg/kg dose to a 10 mg/kg dose in rat. Inclusion of an enzymatically degraded ester linkage can improve the degradation and metabolism profile of the cationic component, while still maintaining the activity of the reLNP formulation. The ester linkage can be internally located within the lipid chain or it may be terminally located at the terminal end of the lipid chain. The internal ester linkage may replace any carbon in the lipid chain.

[00301] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon. Non-limiting examples of reLNPs include,


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and


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[00302] In one embodiment, an immune response may be elicited by delivering a lipid nanoparticle which may include a nanospecies, a polymer and an immunogen. (U.S. Publication No. 20120189700 and International Publication No. WO2012099805; each of which is herein incorporated by reference in their entirety). The polymer may encapsulate the nanospecies or partially encapsulate the nanospecies.

[00303] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier. Mucus is located on mucosal tissue such as, but not limted to, oral (e.g., the buccal and esophageal membranes and tonsil tissue), ophthalmic, gastrointestinal (e.g., stomach, small intestine, large intestine, colon, rectum), nasal, respiratory (e.g., nasal, pharyngeal, tracheal and bronchial membranes), genital (e.g., vaginal, cervical and urethral membranes). Nanoparticles larger than 10-200 nm which are preferred for higher drug encapsulation efficiency and the ability to provide the sustained delivery of a wide array of drugs have been thought to be too large to rapidly diffuse through mucosal barriers. Mucus is continuously secreted, shed, discarded or digested and recycled so most of the trapped particles may be removed from the mucosla tissue within seconds or within a few hours. Large polymeric nanoparticles (200nm -500nm in diameter) which have been coated densely with a low molecular weight polyethylene glycol (PEG) diffused through mucus only 4 to 6-fold lower than the

same particles diffusing in water (Lai et al. PNAS 2007 104(5):1482-487; Lai et al. Adv Drug Deliv Rev. 2009 61(2): 158-171; each of which is herein incorporated by reference in their entirety). The transport of nanoparticles may be determined using rates of permeation and/or fluorescent microscopy techniques including, but not limited to, fluorescence recovery after photobleaching (FRAP) and high resolution multiple particle tracking (MPT).

[00304] The lipid nanoparticle engineered to penetrate mucus may comprise a polymeric material (i.e. a polymeric core) and/or a polymer-vitamin conjugate and/or a tri-block co-polymer. The polymeric material may include, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbamates, polyureas, polycarbonates, poly(styrenes), polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyeneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates. The polymeric material may be biodegradable and/or biocompatible. Non-limiting examples of specific polymers include poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA), poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(L-lactic acid-co-glycolic acid) (PLLGA), poly(D,L-lactide) (PDLA), poly(L-lactide) (PLLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide), polyalkyl cyanoacralate, polyurethane, poly-L-lysine (PLL), hydroxypropyl methacrylate (HPMA), polyethyleneglycol, poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, polyorthoesters, poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyalkylenes such as polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol) (PEG), polyalkylene oxides (PEO), polyalkylene terephthalates such as poly(ethylene terephthalate), polyvinyl alcohols (PVA), polyvinyl ethers, polyvinyl esters such as poly(vinyl acetate), polyvinyl halides such as poly(vinyl chloride) (PVC), polyvinylpyrrolidone, polysiloxanes, polystyrene (PS), polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, hydroxypropylcellulose, carboxymethylcellulose, polymers of acrylic acids, such as

poly(methyl(meth)acrylate) (PMMA), poly(ethyl(meth)acrylate), poly(butyl(meth)acrylate), poly(isobutyl(meth)acrylate), poly(hexyl(meth)acrylate), poly(isodecyl(meth)acrylate), poly(lauryl(meth)acrylate), poly(phenyl(meth)acrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) and copolymers and mixtures thereof, polydioxanone and its copolymers, polyhydroxyalkanoates, polypropylene fumarate, polyoxymethylene, poloxamers, poly(ortho)esters, poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), and trimethylene carbonate, polyvinylpyrrolidone.The lipid nanoparticle may be coated or associated with a co-polymer such as, but not limited to, a block co-polymer, and (poly(ethylene glycol))-(poly(propylene oxide))-(poly(ethylene glycol)) triblock copolymer (see US Publication 20120121718 and US Publication 20100003337; each of which is herein incorporated by reference in their entirety). The co-polymer may be a polymer that is generally regarded as safe (GRAS) and the formation of the lipid nanoparticle may be in such a way that no new chemical entities are created. For example, the lipid nanoparticle may comprise poloxamers coating PLGA nanoparticles without forming new chemical entities which are still able to rapidly penetrate human mucus (Yang et al. Angew. Chem. Int. Ed. 2011 50:2597-2600; herein incorporated by reference in its entirety).

[00305] The vitamin of the polymer-vitamin conjugate may be vitamin E. The vitamin portion of the conjugate may be substituted with other suitable components such as, but not limited to, vitamin A, vitamin E, other vitamins, cholesterol, a hydrophobic moiety, or a hydrophobic component of other surfactants (e.g., sterol chains, fatty acids, hydrocarbon chains and alkylene oxide chains).

[00306] The lipid nanoparticle engineered to penetrate mucus may include surface altering agents such as, but not limited to, mmRNA, anionic protein (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as for example dimethyldioctadecyl-ammonium bromide), sugars or sugar derivatives (e.g., cyclodextrin), nucleic acids, polymers (e.g., heparin, polyethylene glycol and poloxamer), mucolytic agents (e.g., N-acetylcysteine, mugwort, bromelain, papain, clerodendrum, acetylcysteine, bromhexine, carbocisteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, letosteine, stepronin, tiopronin, gelsolin, thymosin β4

dornase alfa, neltenexine, erdosteine) and various DNases including rhDNase. The surface altering agent may be embedded or enmeshed in the particle's surface or disposed (e.g., by coating, adsorption, covalent linkage, or other process) on the surface of the lipid nanoparticle. (see US Publication 20100215580 and US Publication 20080166414; each of which is herein incorporated by reference in their entirety).

[00307] The mucus penetrating lipid nanoparticles may comprise at least one mmRNA described herein. The mmRNA may be encapsulated in the lipid nanoparticle and/or disposed on the surface of the paricle. The mmRNA may be covalently coupled to the lipid nanoparticle. Formulations of mucus penetrating lipid nanoparticles may comprise a plurality of nanoparticles. Further, the formulations may contain particles which may interact with the mucus and alter the structural and/or adhesive properties of the surrounding mucus to decrease mucoadhesion which may increase the delivery of the mucus penetrating lipid nanoparticles to the mucosal tissue.

[00308] In one embodiment, the polynucleotide, primary construct, or mmRNA is formulated as a lipoplex, such as, without limitation, the ATUPLEX™ system, the DACC system, the DBTC system and other siRNA-lipoplex technology from Silence Therapeutics (London, United Kingdom), STEMFECT™ from STEMGENT® (Cambridge, MA), and polyethylenimine (PEI) or protamine-based targeted and non-targeted delivery of nucleic acids acids (Aleku et al. Cancer Res. 2008 68:9788-9798; Strumberg et al. Int J Clin Pharmacol Ther 2012 50:76-78; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Kaufmann et al. Microvasc Res 2010 80:286-293Weide et al. J Immunother. 2009 32:498-507; Weide et al. J Immunother. 2008 31:180-188; Pascolo Expert Opin. Biol. Ther. 4:1285-1294; Fotin-Mleczek et al., 2011 J. Immunother. 34:1-15; Song et al., Nature Biotechnol. 2005, 23:709-717; Peer et al., Proc Natl Acad Sci U S A. 2007 6;104:4095-4100; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein by reference in its entirety).

[00309] In one embodiment such formulations may also be constructed or compositions altered such that they passively or actively are directed to different cell types *in vivo*, including but not limited to hepatocytes, immune cells, tumor cells, endothelial cells, antigen presenting cells, and leukocytes (Akinc et al. Mol Ther. 2010 18:1357-1364; Song et al., Nat Biotechnol. 2005 23:709-717; Judge et al., J Clin Invest. 2009 119:661-673; Kaufmann et al., Microvasc Res 2010 80:286-293; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Basha et al., Mol. Ther. 2011 19:2186-2200; Fenske and Cullis, Expert Opin Drug Deliv. 2008 5:25-44; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety). One example of passive targeting of formulations to liver cells includes the DLin-DMA, DLin-KC2-DMA and MC3-based lipid nanoparticle formulations which have been shown to bind to apolipoprotein E and promote binding and uptake of these formulations into hepatocytes in vivo (Akinc et al. Mol Ther. 2010 18:1357-1364; herein incorporated by reference in its entirety). Formulations can also be selectively targeted through expression of different ligands on their surface as exemplified by, but not limited by, folate, transferrin, N-acetylgalactosamine (GalNAc), and antibody targeted approaches (Kolhatkar et al., Curr Drug Discov Technol. 2011 8:197-206; Musacchio and Torchilin, *Front Biosci.* 2011 16:1388-1412; Yu et al., Mol Membr Biol. 2010 27:286-298; Patil et al., Crit Rev Ther Drug Carrier Syst. 2008 25:1-61; Benoit et al., Biomacromolecules. 2011 12:2708-2714Zhao et al., Expert Opin Drug Deliv. 2008 5:309-319; Akinc et al., Mol Ther. 2010 18:1357-1364; Srinivasan et al., Methods Mol Biol. 2012 820:105-116; Ben-Arie et al., Methods Mol Biol. 2012 757:497-507; Peer 2010 J Control Release. 20:63-68; Peer et al., Proc Natl Acad Sci U S A. 2007 104:4095-4100; Kim et al., Methods Mol Biol. 2011 721:339-353; Subramanya et al., Mol Ther. 2010 18:2028-2037; Song et al., Nat Biotechnol. 2005 23:709-717; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety)..

[00310] In one embodiment, the polynucleotide, primary construct, or mmRNA is formulated as a solid lipid nanoparticle. A solid lipid nanoparticle (SLN) may be

spherical with an average diameter between 10 to 1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic molecules and may be stabilized with surfactants and/or emulsifiers. In a further embodiment, the lipid nanoparticle may be a self-assembly lipid-polymer nanoparticle (see Zhang et al., ACS Nano, 2008, 2 (8), pp 1696–1702; herein incorporated by reference in its entirety).

[00311] Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of polynucleotide, primary construct, or mmRNA directed protein production as these formulations may be able to increase cell transfection by the polynucleotide, primary construct, or mmRNA; and/or increase the translation of encoded protein. One such example involves the use of lipid encapsulation to enable the effective systemic delivery of polyplex plasmid DNA (Heyes et al., Mol Ther. 2007 15:713-720; herein incorporated by reference in its entirety). The liposomes, lipoplexes, or lipid nanoparticles may also be used to increase the stability of the polynucleotide, primary construct, or mmRNA.

[00312] In one embodiment, the the polynucleotides, primary constructs, and/or the mmRNA of the present invention can be formulated for controlled release and/or targeted delivery. As used herein, "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome. In one embodiment, the polynucleotides, primary constructs or the mmRNA may be encapsulated into a delivery agent described herein and/or known in the art for controlled release and/or targeted delivery. As used herein, the term "encapsulate" means to enclose, surround or encase. As it relates to the formulation of the compounds of the invention, encapsulation may be substantial, complete or partial. The term "substitantially encapsulated" means that at least greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.9 or greater than 99.999% of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. "Partially encapsulation" means that less than 10, 10, 20, 30, 40 50 or less of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. Advantageously, encapsulation may be determined by measuring the escape or the activity of the

pharmaceutical composition or compound of the invention using fluorescence and/or electron micrograph. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the pharmaceutical composition or compound of the invention are encapsulated in the delivery agent.

[00313] In another embodiment, the polynucleotides, primary constructs, or the mmRNA may be encapsulated into a lipid nanoparticle or a rapidly eliminating lipid nanoparticle and the lipid nanoparticles or a rapidly eliminating lipid nanoparticle may then be encapsulated into a polymer, hydrogel and/or surgical sealant described herein and/or known in the art. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[00314] In one embodiment, the lipid nanoparticle may be encapsulated into any polymer or hydrogel known in the art which may form a gel when injected into a subject. As another non-limiting example, the lipid nanoparticle may be encapsulated into a polymer matrix which may be biodegradable.

[00315] In one embodiment, the polynucleotide, primary construct, or mmRNA formulation for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®).

[00316] In one embodiment, the controlled release and/or targeted delivery formulation may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and

combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[00317] In one embodiment, the polynucleotides, primary constructs, and/or the mmRNA of the present invention may be encapsulated in a therapeutic nanoparticle. Therapeutic nanoparticles may be formulated by methods described herein and known in the art such as, but not limited to, International Pub Nos. WO2010005740, WO2010030763, WO2010005721, WO2010005723, WO2012054923, US Pub. Nos. US20110262491, US20100104645, US20100087337, US20100068285, US20110274759, US20100068286, and US Pat No. 8,206,747; each of which is herein incorporated by reference in their entirety. In another embodiment, therapeutic polymer nanoparticles may be identified by the methods described in US Pub No. US20120140790, herein incorporated by reference in its entirety.

[00318] In one embodiment, the therapeutic nanoparticle of may be formulated for sustained release. As used herein, "sustained release" refers to a pharmaceutical composition or compound that conforms to a release rate over a specific period of time. The period of time may include, but is not limited to, hours, days, weeks, months and years. As a non-limiting example, the sustained release nanoparticle may comprise a polymer and a therapeutic agent such as, but not limited to, the the polynucleotides, primary constructs, and mmRNA of the present invention (see International Pub No. 2010075072 and US Pub No. US20100216804 and US20110217377, each of which is herein incorporated by reference in their entirety).

[00319] In one embodiment, the therapeutic nanoparticles may be formulated to be target specific. As a non-limiting example, the thereapeutic nanoparticles may include a corticosteroid (see International Pub. No. WO2011084518). In one embodiment, the therapeutic nanoparticles may be formulated to be cancer specific. As a non-limiting example, the therapeutic nanoparticles may be formulated in nanoparticles described in International Pub No. WO2008121949, WO2010005726, WO2010005725, WO2011084521 and US Pub No. US20100069426, US20120004293 and US20100104655, each of which is herein incorporated by reference in their entirety.

[00320] In one embodiment, the nanoparticles of the present invention may comprise a polymeric matrix. As a non-limiting example, the nanoparticle may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[00321] In one embodiment, the diblock copolymer may include PEG in combination with a polymer such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[00322] In one embodiment, the therapeutic nanoparticle comprises a diblock copolymer. As a non-limiting example the therapeutic nanoparticle comprises a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, herein incorporated by reference in their entireties). In another non-limiting example, the therapeutic nanoparticle is a stealth nanoparticle comprising a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, each of which is herein incorporated by reference in its entirety).

[00323] In one embodiment, the therapeutic nanoparticle may comprise at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[00324] In one embodiment, the therapeutic nanoparticles may comprise at least one cationic polymer described herein and/or known in the art.

[00325] In one embodiment, the therapeutic nanoparticles may comprise at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers and combinations thereof.

[00326] In one embodiment, the therapeutic nanoparticles may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[00327] In another embodiment, the therapeutic nanoparticle may include a conjugation of at least one targeting ligand.

[00328] In one embodiment, the therapeutic nanoparticle may be formulated in an aqueous solution which may be used to target cancer (see International Pub No. WO2011084513 and US Pub No. US20110294717, each of which is herein incorporated by reference in their entirety).

[00329] In one embodiment, the polynucleotides, primary constructs, or mmRNA may be encapsulated in, linked to and/or associated with synthetic nanocarriers. The synthetic nanocarriers may be formulated using methods known in the art and/or described herein. As a non-limiting example, the synthetic nanocarriers may be formulated by the methods described in International Pub Nos. WO2010005740, WO2010030763 and US Pub. Nos. US20110262491, US20100104645 and US20100087337, each of which is herein incorporated by reference in their entirety. In another embodiment, the synthetic nanocarrier formulations may be lyophilized by methods described in International Pub. No. WO2011072218 and US Pat No. 8,211,473; each of which is herein incorporated by reference in their entireties.

[00330] In one embodiment, the synthetic nanocarriers may contain reactive groups to release the polynucleotides, primary constructs and/or mmRNA described herein (see International Pub. No. WO20120952552 and US Pub No. US20120171229, each of which is herein incorporated by reference in their entirety).

[00331] In one embodiment, the synthetic nanocarriers may contain an immunostimulatory agent to enhance the immune response from delivery of the synthetic nanocarrier. As a non-limiting example, the synthetic nanocarrier may comprise a Th1 immunostimulatory agent which may enhance a Th1-based response of the immune system (see International Pub No. WO2010123569 and US Pub. No. US20110223201, each of which is herein incorporated by reference in its entirety).

[00332] In one embodiment, the synthetic nanocarriers may be formulated for targeted release. In one embodiment, the synthetic nanocarrier is formulated to release the polynucleotides, primary constructs and/or mmRNA at a specified pH and/or after a desired time interval. As a non-limiting example, the synthetic nanoparticle may be formulated to release the polynucleotides, primary constructs and/or mmRNA after 24 hours and/or at a pH of 4.5 (see International Pub. Nos. WO2010138193 and WO2010138194 and US Pub Nos. US20110020388 and US20110027217, each of which is herein incorporated by reference in their entireties).

[00333] In one embodiment, the synthetic nanocarriers may be formulated for controlled and/or sustained release of the polynucleotides, primary constructs and/or mmRNA described herein. As a non-limiting example, the synthetic nanocarriers for sustained release may be formulated by methods known in the art, described herein and/or as described in International Pub No. WO2010138192 and US Pub No. 20100303850, each of which is herein incorporated by reference in their entireties.

Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles

[00334] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using natural and/or synthetic polymers. Non-limiting examples of polymers which may be used for delivery include, but are not limited to, Dynamic POLYCONJUGATE™ formulations from MIRUS® Bio (Madison, WI) and Roche Madison (Madison, WI), PHASERX™ polymer formulations such as, without limitation, SMARTT POLYMER TECHNOLOGY™ (Seattle, WA), DMRI/DOPE, poloxamer, VAXFECTIN® adjuvant from Vical (San Diego, CA), chitosan, cyclodextrin from Calando Pharmaceuticals (Pasadena, CA), dendrimers and poly(lactic-co-glycolic acid) (PLGA) polymers. RONDEL™ (RNAi/Oligonucleotide

Nanoparticle Delivery) polymers (Arrowhead Research Corporation, Pasadena, CA) and pH responsive co-block polymers such as, but not limited to, PHASERX™ (Seattle, WA).

[00335] A non-limiting example of PLGA formulations include, but are not limited to, PLGA injectable depots (e.g., ELIGARD® which is formed by dissolving PLGA in 66% N-methyl-2-pyrrolidone (NMP) and the remainder being aqueous solvent and leuprolide. Once injected, the PLGA and leuprolide peptide precipitates into the subcutaneous space).

[00336] Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides *in vivo* into the cell cytoplasm (reviewed in deFougerolles *Hum Gene Ther.* 2008 19:125-132; herein incorporated by reference in its entirety). Two polymer approaches that have yielded robust *in vivo* delivery of nucleic acids, in this case with small interfering RNA (siRNA), are dynamic polyconjugates and cyclodextrin-based nanoparticles. The first of these delivery approaches uses dynamic polyconjugates and has been shown *in vivo* in mice to effectively deliver siRNA and silence endogenous target mRNA in hepatocytes (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887). This particular approach is a multicomponent polymer system whose key features include a membrane-active polymer to which nucleic acid, in this case siRNA, is covalently coupled via a disulfide bond and where both PEG (for charge masking) and *N*-acetylgalactosamine (for hepatocyte targeting) groups are linked via pH-sensitive bonds (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887). On binding to the hepatocyte and entry into the endosome, the polymer complex disassembles in the low-pH environment, with the polymer exposing its positive charge, leading to endosomal escape and cytoplasmic release of the siRNA from the polymer. Through replacement of the *N*-acetylgalactosamine group with a mannose group, it was shown one could alter targeting from asialoglycoprotein receptor-expressing hepatocytes to sinusoidal endothelium and Kupffer cells. Another polymer approach involves using transferrin-targeted cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the *EWS-FLI1* gene product in transferrin receptor-expressing Ewing's sarcoma tumor cells (Hu-Lieskovan *et al.*, Cancer

Res.2005 65: 8984-8982) and siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel *et al.*, Proc Natl Acad Sci USA 2007 104:5715-21). Both of these delivery strategies incorporate rational approaches using both targeted delivery and endosomal escape mechanisms.

[00337] The polymer formulation can permit the sustained or delayed release of the polynucleotide, primary construct, or mmRNA (e.g., following intramuscular or subcutaneous injection). The altered release profile for the polynucleotide, primary construct, or mmRNA can result in, for example, translation of an encoded protein over an extended period of time. The polymer formulation may also be used to increase the stability of the polynucleotide, primary construct, or mmRNA. Biodegradable polymers have been previously used to protect nucleic acids other than mmRNA from degradation and been shown to result in sustained release of payloads in vivo (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Sullivan et al., Expert Opin Drug Deliv. 2010 7:1433-1446; Convertine et al., Biomacromolecules. 2010 Oct 1; Chu et al., Acc Chem Res. 2012 Jan 13; Manganiello et al., Biomaterials. 2012 33:2301-2309; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Singha et al., Nucleic Acid Ther. 2011 2:133-147; deFougerolles Hum Gene Ther. 2008 19:125-132; Schaffert and Wagner, Gene Ther. 2008 16:1131-1138; Chaturvedi et al., Expert Opin Drug Deliv. 2011 8:1455-1468; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[00338] In one embodiment, the pharmaceutical compositions may be sustained release formulations. In a further embodiment, the sustained release formulations may be for subcutaneous delivery. Sustained release formulations may include, but are not limited to, PLGA microspheres, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[00339] As a non-limiting example modified mRNA may be formulated in PLGA microspheres by preparing the PLGA microspheres with tunable release rates (e.g.,

days and weeks) and encapsulating the modified mRNA in the PLGA microspheres while maintaining the integrity of the modified mRNA during the encapsulation process. EVAc are non-biodegradeable, biocompatible polymers which are used extensively in pre-clinical sustained release implant applications (e.g., extended release products Ocusert a pilocarpine ophthalmic insert for glaucoma or progestasert a sustained release progesterone intrauterine deivce; transdermal delivery systems Testoderm, Duragesic and Selegiline; catheters). Poloxamer F-407 NF is a hydrophilic, non-ionic surfactant triblock copolymer of polyoxyethylene-polyoxypropylene-polyoxyethylene having a low viscosity at temperatures less than 5°C and forms a solid gel at temperatures greater than 15°C. PEG-based surgical sealants comprise two synthetic PEG components mixed in a delivery device which can be prepared in one minute, seals in 3 minutes and is reabsorbed within 30 days. GELSITE® and natural polymers are capable of in-situ gelation at the site of administration. They have been shown to interact with protein and peptide therapeutic candidates through ionic ineraction to provide a stabilizing effect.

[00340] Polymer formulations can also be selectively targeted through expression of different ligands as exemplified by, but not limited by, folate, transferrin, and N-acetylgalactosamine (GalNAc) (Benoit et al., Biomacromolecules. 2011 12:2708-2714; Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[00341] The polynucleotides, primary constructs and/or mmRNA of the invention may be formulated with or in a polymeric compound. The polymer may include at least one polymer such as, but not limited to, polyethenes, polyethylene glycol (PEG), poly(l-lysine)(PLL), PEG grafted to PLL, cationic lipopolymer, biodegradable cationic lipopolymer, polyethyleneimine (PEI), cross-linked branched poly(alkylene imines), a polyamine derivative, a modified poloxamer, a biodegradable polymer, biodegradable block copolymer, biodegradable random copolymer, biodegradable polyester copolymer, biodegradable polyester block copolymer, biodegradable polyester block random copolymer, linear biodegradable copolymer, poly[α-(4-aminobutyl)-L-glycolic acid) (PAGA), biodegradable cross-linked cationic multi-

block copolymers, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), acrylic polymers, amine-containing polymers or combinations thereof .

[00342] As a non-limiting example, the polynucleotides, primary constructs and/or mmRNA of the invention may be formulated with the polymeric compound of PEG grafted with PLL as described in U.S. Pat. No. 6,177,274 herein incorporated by reference in its entirety. The formulation may be used for transfecting cells *in vitro* or for *in vivo* delivery of the polynucleotides, primary constructs and/or mmRNA. In another example, the polynucleotides, primary constructs and/or mmRNA may be suspended in a solution or medium with a cationic polymer, in a dry pharmaceutical composition or in a solution that is capable of being dried as described in U.S. Pub. Nos. 20090042829 and 20090042825 each of which are herein incorporated by reference in their entireties.

[00343] As another non-limiting example the polynucleotides, primary constructs or mmRNA of the invention may be formulated with a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, each of which is herein incorporated by reference in their entireties). As a non-limiting example, the polynucleotides, primary constructs or mmRNA of the invention may be formulated with a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[00344] A polyamine derivative may be used to deliver nucleic acids or to treat and/or prevent a disease or to be included in an implantable or injectable device (U.S. Pub. No. 20100260817 herein incorporated by reference in its entirety). As a non-limiting example, a pharmaceutical composition may include the modified nucleic acids and mmRNA and the polyamine derivative described in U.S. Pub. No. 20100260817 (the contents of which are incorporated herein by reference in its entirety.

[00345] The polynucleotides, primary constructs or mmRNA of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[00346] In one embodiment, the polynucleotides, primary constructs or mmRNA of the present invention may be formulated with at least one polymer described in International Publication Nos. WO2011115862, WO2012082574 and WO2012068187, each of which is herein incorporated by reference in their entireties. In another embodiment the polynucleotides, primary constructs or mmRNA of the present invention may be formulated with a polymer of formula Z as described in WO2011115862, herein incorporated by reference in its entirety. In yet another embodiment, the polynucleotides, primary constructs or mmRNA may be formulated with a polymer of formula Z, Z' or Z'' as described in WO2012082574 or WO2012068187, each of which are herein incorporated by reference in their entireties. The polymers formulated with the modified RNA of the present invention may be synthesized by the methods described in WO2012082574 or WO2012068187, each of which is herein incorporated by reference in their entireties.

[00347] Formulations of polynucleotides, primary constructs or mmRNA of the invention may include at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers or combinations thereof.

[00348] For example, the polynucleotides, primary constructs and/or mmRNA of the invention may be formulated in a pharmaceutical compound including a poly(alkylene imine), a biodegradable cationic lipopolymer, a biodegradable block copolymer, a biodegradable polymer, or a biodegradable random copolymer, a biodegradable polyester block copolymer, a biodegradable polyester polymer, a biodegradable polyester random copolymer, a linear biodegradable copolymer, PAGA, a biodegradable cross-linked cationic multi-block copolymer or combinations thereof. The biodegradable cationic lipopolymer may be made my methods known in

the art and/or described in U.S. Pat. No. 6,696,038, U.S. App. Nos. 20030073619 and 20040142474 each of which is herein incorporated by reference in their entireties.The poly(alkylene imine) may be made using methods known in the art and/or as described in U.S. Pub. No. 20100004315, herein incorporated by reference in its entirety. The biodegradabale polymer, biodegradable block copolymer, the biodegradable random copolymer, biodegradable polyester block copolymer, biodegradable polyester polymer, or biodegradable polyester random copolymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,517,869 and 6,267,987, the contents of which are each incorporated herein by reference in its entirety. The linear biodegradable copolymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,652,886. The PAGA polymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,217,912 herein incorporated by reference in its entirety. The PAGA polymer may be copolymerized to form a copolymer or block copolymer with polymers such as but not limited to, poly-L-lysine, polyargine, polyornithine, histones, avidin, protamines, polylactides and poly(lactide-co-glycolides). The biodegradable cross-linked cationic multi-block copolymers may be made my methods known in the art and/or as described in U.S. Pat. No. 8,057,821 or U.S. Pub. No. 2012009145 each of which is herein incorporated by reference in their entireties. For example, the multi-block copolymers may be synthesized using linear polyethyleneimine (LPEI) blocks which have distinct patterns as compared to branched polyethyleneimines. Further, the composition or pharmaceutical composition may be made by the methods known in the art, described herein, or as described in U.S. Pub. No. 20100004315 or U.S. Pat. Nos. 6,267,987 and 6,217,912 each of which is herein incorporated by reference in their entireties.

[00349] The polynucleotides, primary constructs, and mmRNA of the invention may be formulated with at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[00350] In one embodiment, the polymers described herein may be conjugated to a lipid-terminating PEG. As a non-limiting example, PLGA may be conjugated to a lipid-terminating PEG forming PLGA-DSPE-PEG. As another non-limiting example, PEG conjugates for use with the present invention are described in International Publication No. WO2008103276, herein incorporated by reference in its entirety.

[00351] In one embodiment, the polynucleotides, primary constructs and/or mmRNA described herein may be conjugated with another compound. Non-limiting examples of conjugates are described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. In another embodiment, , the polynucleotides, primary constructs and/or mmRNA of the present invention may be conjugated with conjugates of formula 1-122 as described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties.

[00352] As described in U.S. Pub. No. 20100004313, herein incorporated by reference in its entirety, a gene delivery composition may include a nucleotide sequence and a poloxamer. For example, the polynucleotide, primary construct and/or mmRNA of the present inveition may be used in a gene delivery composition with the poloxamer described in U.S. Pub. No. 20100004313.

[00353] In one embodiment, the polymer formulation of the present invention may be stabilized by contacting the polymer formulation, which may include a cationic carrier, with a cationic lipopolymer which may be covalently linked to cholesterol and polyethylene glycol groups. The polymer formulation may be contacted with a cationic lipopolymer using the methods described in U.S. Pub. No. 20090042829 herein incorporated by reference in its entirety. The cationic carrier may include, but is not limited to, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane(DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-

dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N′,N′-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl) diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleyl-N,N-dimethylammonium chloride DODAC) and combinations thereof.

[00354] The polynucleotide, primary construct, and mmRNA of the invention can also be formulated as a nanoparticle using a combination of polymers, lipids, and/or other biodegradable agents, such as, but not limited to, calcium phosphate. Components may be combined in a core-shell, hybrid, and/or layer-by-layer architecture, to allow for fine-tuning of the nanoparticle so to delivery of the polynucleotide, primary construct and mmRNA may be enhanced (Wang et al., Nat Mater. 2006 5:791-796; Fuller et al., Biomaterials. 2008 29:1526-1532; DeKoker et al., Adv Drug Deliv Rev. 2011 63:748-761; Endres et al., Biomaterials. 2011 32:7721-7731; Su et al., Mol Pharm. 2011 Jun 6;8(3):774-87; herein incorporated by reference in its entirety).

[00355] Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver polynucleotides, primary constructs and mmRNA *in vivo*. In one embodiment, a lipid coated calcium phosphate nanoparticle, which may also contain a targeting ligand such as anisamide, may be used to deliver the polynucleotide, primary construct and mmRNA of the present invention. For example, to effectively deliver siRNA in a mouse metastatic lung model a lipid coated calcium phosphate nanoparticle was used (Li et al., J Contr Rel. 2010 142: 416-421; Li et al., J Contr Rel. 2012 158:108-114; Yang et al., Mol Ther. 2012 20:609-615). This delivery system combines both a targeted nanoparticle and a component to enhance the endosomal escape, calcium phosphate, in order to improve delivery of the siRNA.

[00356] In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to delivery polynucleotides, primary constructs and mmRNA

(Kazikawa et al., J Contr Rel. 2004 97:345-356; Kazikawa et al., J Contr Rel. 2006 111:368-370).

[00357] In one embodiment, a PEG-charge-conversional polymer (Pitella et al., Biomaterials. 2011 32:3106-3114) may be used to form a nanoparticle to deliver the polynucleotides, primary constructs and mmRNA of the present invention. The PEG-charge-conversional polymer may improve upon the PEG-polyanion block copolymers by being cleaved into a polycation at acidic pH, thus enhancing endosomal escape.

[00358] The use of core-shell nanoparticles has additionally focused on a high-throughput approach to synthesize cationic cross-linked nanogel cores and various shells (Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-13001). The complexation, delivery, and internalization of the polymeric nanoparticles can be precisely controlled by altering the chemical composition in both the core and shell components of the nanoparticle. For example, the core-shell nanoparticles may efficiently deliver siRNA to mouse hepatocytes after they covalently attach cholesterol to the nanoparticle.

[00359] In one embodiment, a hollow lipid core comprising a middle PLGA layer and an outer neutral lipid layer containg PEG may be used to delivery of the polynucleotide, primary construct and mmRNA of the present invention. As a non-limiting example, in mice bearing a luciferease-expressing tumor, it was determined that the lipid-polymer-lipid hybrid nanoparticle significantly suppressed luciferase expression, as compared to a conventional lipoplex (Shi et al, Angew Chem Int Ed. 2011 50:7027-7031).

Peptides and Proteins

[00360] The polynucleotide, primary construct, and mmRNA of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the polynucleotide, primary construct, or mmRNA. In one embodiment, peptides such as, but not limited to, cell penetrating peptides and proteins and peptides that enable intracellular delivery may be used to deliver pharmaceutical formulations. A non-limiting example of a cell penetrating peptide which may be used with the pharmaceutical formulations of the present invention includes a cell-penetrating

peptide sequence attached to polycations that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratins, transportans, or hCT derived cell-penetrating peptides (see, e.g., Caron et al., Mol. Ther. 3(3):310-8 (2001); Langel, Cell-Penetrating Peptides: Processes and Applications (CRC Press, Boca Raton FL, 2002); El-Andaloussi et al., Curr. Pharm. Des. 11(28):3597-611 (2003); and Deshayes et al., Cell. Mol. Life Sci. 62(16):1839-49 (2005), all of which are incorporated herein by reference). The compositions can also be formulated to include a cell penetrating agent, e.g., liposomes, which enhance delivery of the compositions to the intracellular space. polynucleotides, primary constructs, and mmRNA of the invention may be complexed to peptides and/or proteins such as, but not limited to, peptides and/or proteins from Aileron Therapeutics (Cambridge, MA) and Permeon Biologics (Cambridge, MA) in order to enable intracellular delivery (Cronican et al., ACS Chem. Biol. 2010 5:747-752; McNaughton et al., Proc. Natl. Acad. Sci. USA 2009 106:6111-6116; Sawyer, Chem Biol Drug Des. 2009 73:3-6; Verdine and Hilinski, Methods Enzymol. 2012;503:3-33; all of which are herein incorporated by reference in its entirety).

[00361] In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the polynucleotide, primary construct, or mmRNA may be introduced.

[00362] Formulations of the including peptides or proteins may be used to increase cell transfection by the polynucleotide, primary construct, or mmRNA, alter the biodistribution of the polynucleotide, primary construct, or mmRNA (e.g., by targeting specific tissues or cell types), and/or increase the translation of encoded protein.

Cells

[00363] The polynucleotide, primary construct, and mmRNA of the invention can be transfected *ex vivo* into cells, which are subsequently transplanted into a subject. As non-limiting examples, the pharmaceutical compositions may include red blood cells to deliver modified RNA to liver and myeloid cells, virosomes to deliver modified RNA in virus-like particles (VLPs), and electroporated cells such as, but not limited to, from MAXCYTE® (Gaithersburg, MD) and from ERYTECH® (Lyon, France) to deliver modified RNA. Examples of use of red blood cells, viral particles and electroporated cells to deliver payloads other than mmRNA have been documented (Godfrin et al., Expert Opin Biol Ther. 2012 12:127-133; Fang et al., Expert Opin Biol Ther. 2012 12:385-389; Hu et al., Proc Natl Acad Sci U S A. 2011 108:10980-10985; Lund et al., Pharm Res. 2010 27:400-420; Huckriede et al., J Liposome Res. 2007;17:39-47; Cusi, Hum Vaccin. 2006 2:1-7; de Jonge et al., Gene Ther. 2006 13:400-411; all of which are herein incorporated by reference in its entirety).

[00364] The polynucleotides, primary constructs and mmRNA may be delivered in synthetic VLPs synthesized by the methods described in International Pub No. WO2011085231 and US Pub No. 20110171248, each of which is herein incorporated by reference in their entireties.

[00365] Cell-based formulations of the polynucleotide, primary construct, and mmRNA of the invention may be used to ensure cell transfection (e.g., in the cellular carrier), alter the biodistribution of the polynucleotide, primary construct, or mmRNA (e.g., by targeting the cell carrier to specific tissues or cell types), and/or increase the translation of encoded protein.

[00366] A variety of methods are known in the art and suitable for introduction of nucleic acid into a cell, including viral and non-viral mediated techniques. Examples of typical non-viral mediated techniques include, but are not limited to, electroporation, calcium phosphate mediated transfer, nucleofection, sonoporation, heat shock, magnetofection, liposome mediated transfer, microinjection, microprojectile mediated transfer (nanoparticles), cationic polymer mediated transfer (DEAE-dextran, polyethylenimine, polyethylene glycol (PEG) and the like) or cell fusion.

[00367] The technique of sonoporation, or cellular sonication, is the use of sound (e.g., ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. Sonoporation methods are known to those in the art and are used to deliver nucleic acids *in vivo* (Yoon and Park, Expert Opin Drug Deliv. 2010 7:321-330; Postema and Gilja, Curr Pharm Biotechnol. 2007 8:355-361; Newman and Bettinger, Gene Ther. 2007 14:465-475; all herein incorporated by reference in their entirety). Sonoporation methods are known in the art and are also taught for example as it relates to bacteria in US Patent Publication 20100196983 and as it relates to other cell types in, for example, US Patent Publication 20100009424, each of which are incorporated herein by reference in their entirety.

[00368] Electroporation techniques are also well known in the art and are used to deliver nucleic acids *in vivo* and clinically (Andre et al., Curr Gene Ther. 2010 10:267-280; Chiarella et al., Curr Gene Ther. 2010 10:281-286; Hojman, Curr Gene Ther. 2010 10:128-138; all herein incorporated by reference in their entirety). In one embodiment, polynucleotides, primary constructs or mmRNA may be delivered by electroporation as described in Example 26.

Hyaluronidase

[00369] The intramuscular or subcutaneous localized injection of polynucleotide, primary construct, or mmRNA of the invention can include hyaluronidase, which catalyzes the hydrolysis of hyaluronan. By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability (Frost, Expert Opin. Drug Deliv. (2007) 4:427-440; herein incorporated by reference in its entirety). It is useful to speed their dispersion and systemic distribution of encoded proteins produced by transfected cells. Alternatively, the hyaluronidase can be used to increase the number of cells exposed to a polynucleotide, primary construct, or mmRNA of the invention administered intramuscularly or subcutaneously.

Nanoparticle Mimics

[00370] The polynucleotide, primary construct or mmRNA of the invention may be encapsulated within and/or absorbed to a nanoparticle mimic. A nanoparticle mimic can mimic the delivery function organisms or particles such as, but not limited to,

pathogens, viruses, bacteria, fungus, parasites, prions and cells. As a non-limiting example the polynucleotide, primary construct or mmRNA of the invention may be encapsulated in a non-viron particle which can mimic the delivery function of a virus (see International Pub. No. WO2012006376 herein incorporated by reference in its entirety).

Nanotubes

[00371] The polynucleotides, primary constructs or mmRNA of the invention can be attached or otherwise bound to at least one nanotube such as, but not limited to, rosette nanotubes, rosette nanotubes having twin bases with a linker, carbon nanotubes and/or single-walled carbon nanotubes, The polynucleotides, primary constructs or mmRNA may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces.

[00372] In one embodiment, the nanotube can release one or more polynucleotides, primary constructs or mmRNA into cells. The size and/or the surface structure of at least one nanotube may be altered so as to govern the interaction of the nanotubes within the body and/or to attach or bind to the polynucleotides, primary constructs or mmRNA disclosed herein. In one embodiment, the building block and/or the functional groups attached to the building block of the at least one nanotube may be altered to adjust the dimensions and/or properties of the nanotube. As a non-limiting example, the length of the nanotubes may be altered to hinder the nanotubes from passing through the holes in the walls of normal blood vessels but still small enough to pass through the larger holes in the blood vessels of tumor tissue.

[00373] In one embodiment, at least one nanotube may also be coated with delivery enhancing compounds including polymers, such as, but not limited to, polyethylene glycol. In another embodiment, at least one nanotube and/or the polynucleotides, primary constructs or mmRNA may be mixed with pharmaceutically acceptable excipients and/or delivery vehicles.

[00374] In one embodiment, the polynucleotides, primary constructs or mmRNA are attached and/or otherwise bound to at least one rosette nanotube. The rosette nanotubes may be formed by a process known in the art and/or by the process described in International Publication No. WO2012094304, herein incorporated by

reference in its entirety. At least one polynucleotide, primary construct and/or mmRNA may be attached and/or otherwise bound to at least one rosette nanotube by a process as described in International Publication No. WO2012094304, herein incorporated by reference in its entirety, where rosette nanotubes or modules forming rosette nanotubes are mixed in aqueous media with at least one polynucleotide, primary construct and/or mmRNA under conditions which may cause at least one polynucleotide, primary construct or mmRNA to attach or otherwise bind to the rosette nanotubes.

Conjugates

[00375] The polynucleotides, primary constructs, and mmRNA of the invention include conjugates, such as a polynucleotide, primary construct, or mmRNA covalently linked to a carrier or targeting group, or including two encoding regions that together produce a fusion protein (e.g., bearing a targeting group and therapeutic protein or peptide).

[00376] The conjugates of the invention include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[00377] Representative U.S. patents that teach the preparation of polynucleotide conjugates, particularly to RNA, include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference in their entireties.

[00378] In one embodiment, the conjugate of the present invention may function as a carrier for the polynucleotides, primary constructs and/or mmRNA of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylysine, polyalkylenimine, and polyethylenimine which may be grafted to with poly(ethylene glycol). As a non-limiting example, the conjugate may be similar to the polymeric conjugate and the method of synthesizing the polymeric conjugate described in U.S. Pat. No. 6,586,524 herein incorporated by reference in its entirety.

[00379] The conjugates can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer.

[00380] Targeting groups can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody,

that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

[00381] The targeting group can be any ligand that is capable of targeting a specific receptor. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6P, apatamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In particular embodiments, the targeting group is an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[00382] In one embodiment, pharmaceutical compositions of the present invention may include chemical modifications such as, but not limited to, modifications similar to locked nucleic acids.

[00383] Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santaris, include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

[00384] Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

[00385] Some embodiments featured in the invention include polynucleotides, primary constructs or mmRNA with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O—P(O)$_2$--O--

CH_2--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the polynucletotides featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[00386] Modifications at the 2′ position may also aid in delivery. Preferably, modifications at the 2′ position are not located in a polypeptide-coding sequence, i.e., not in a translatable region. Modifications at the 2′ position may be located in a 5′UTR, a 3′UTR and/or a tailing region. Modifications at the 2′ position can include one of the following at the 2' position: H (i.e., 2′-deoxy); F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, the polynucleotides, primary constructs or mmRNA include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties, or a group for improving the pharmacodynamic properties, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--$N(CH_2)_2$, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions, particularly the 3' position of the

sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. polynucleotides of the invention may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920 and each of which is herein incorporated by reference.

[00387] In still other embodiments, the polynucleotide, primary construct, or mmRNA is covalently conjugated to a cell penetrating polypeptide. The cell-penetrating peptide may also include a signal sequence. The conjugates of the invention can be designed to have increased stability; increased cell transfection; and/or altered the biodistribution (e.g., targeted to specific tissues or cell types).

Self-Assembled Nucleic Acid Nanoparticles

[00388] Self-assembled nanoparticles have a well-defined size which may be precisely controlled as the nucleic acid strands may be easily reprogrammable. For example, the optimal particle size for a cancer-targeting nanodelivery carrier is 20-100 nm as a diameter greater than 20 nm avoids renal clearance and enhances delivery to certain tumors through enhanced permeability and retention effect. Using self-assembled nucleic acid nanoparticles a single uniform population in size and shape having a precisely controlled spatial orientation and density of cancer-targeting ligands for enhanced delivery. As a non-limiting example, oligonucleotide nanoparticles are prepared using programmable self-assembly of short DNA fragments and therapeutic siRNAs. These nanoparticles are molecularly identical with controllable particle size and target ligand location and density. The DNA fragments and siRNAs self-assembled into a one-step reaction to generate DNA/siRNA tetrahedral nanoparticles for targeted *in vivo* delivery. (Lee et al., Nature Nanotechnology 2012 7:389-393).

Excipients

[00389] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion

media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[00390] In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[00391] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical compositions.

[00392] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, *etc.*, and/or combinations thereof.

[00393] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic

acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, *etc.*, and/or combinations thereof.

[00394] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN®20], polyoxyethylene sorbitan [TWEENn®60], polyoxyethylene sorbitan monooleate [TWEEN®80], sorbitan monopalmitate [SPAN®40], sorbitan monostearate [Span®60], sorbitan tristearate [Span®65], glyceryl monooleate, sorbitan monooleate [SPAN®80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ®45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ®30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate,

PLUORINC®F 68, POLOXAMER®188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

[00395] Exemplary binding agents include, but are not limited to, starch (*e.g.* cornstarch and starch paste); gelatin; sugars (*e.g.* sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol,); natural and synthetic gums (*e.g.* acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum®), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; *etc.*; and combinations thereof.

[00396] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene

glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL®115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®.

[00397] Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, *etc.*, and/or combinations thereof.

[00398] Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, *etc.*, and combinations thereof.

[00399] Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn,

cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and/or combinations thereof.

[00400] Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

Delivery

[00401] The present disclosure encompasses the delivery of polynucleotides, primary constructs or mmRNA for any of therapeutic, pharmaceutical, diagnostic or imaging by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

Naked Delivery

[00402] The polynucleotides, primary constructs or mmRNA of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering polynucleotides, primary constructs or mmRNA free from agents which promote transfection. For example, the polynucleotides, primary constructs or mmRNA delivered to the cell may contain no modifications. The naked polynucleotides, primary constructs or mmRNA may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

[00403] The polynucleotides, primary constructs or mmRNA of the present invention may be formulated, using the methods described herein. The formulations may contain polynucleotides, primary constructs or mmRNA which may be modified and/or unmodified. The formulations may further include, but are not limited to, cell

penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible polymer, a solvent, and a sustained-release delivery depot. The formulated polynucleotides, primary constructs or mmRNA may be delivered to the cell using routes of administration known in the art and described herein.

[00404] The compositions may also be formulated for direct delivery to an organ or tissue in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels, lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with the compositions, and the like.

Administration

[00405] The polynucleotides, primary constructs or mmRNA of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral, gastroenteral, epidural, oral, transdermal, epidural (peridural), intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection, (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), or in ear drops. In specific embodiments, compositions may be administered in a way which allows them cross the blood-brain barrier, vascular barrier, or other epithelial barrier.Non-limiting routes of administration for the polynucleotides, primary constructs or mmRNA of the present invention are described below.

Parenteral and Injectible Administration

[00406] Liquid dosage forms for oral and parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[00407] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[00408] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00409] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular

injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Rectal and Vaginal Administration

[00410] Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing compositions with suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

Oral Administration

[00411] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, an active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.* starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.* carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.* glycerol), disintegrating agents (*e.g.* agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.* paraffin), absorption accelerators (*e.g.* quaternary ammonium compounds), wetting agents (*e.g.* cetyl alcohol and glycerol monostearate), absorbents (*e.g.* kaolin and bentonite clay), and lubricants (*e.g.* talc, calcium stearate, magnesium stearate, solid polyethylene

glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

Topical or Transdermal Administration

[00412] As described herein, compositions containing the polynucleotides, primary constructs or mmRNA of the invention may be formulated for administration topically. The skin may be an ideal target site for delivery as it is readily accessible. Gene expression may be restricted not only to the skin, potentially avoiding nonspecific toxicity, but also to specific layers and cell types within the skin.

[00413] The site of cutaneous expression of the delivered compositions will depend on the route of nucleic acid delivery. Three routes are commonly considered to deliver polynucleotides, primary constructs or mmRNA to the skin: (i) topical application (e.g. for local/regional treatment and/or cosmetic applications); (ii) intradermal injection (e.g. for local/regional treatment and/or cosmetic applications); and (iii) systemic delivery (e.g. for treatment of dermatologic diseases that affect both cutaneous and extracutaneous regions). polynucleotides, primary constructs or mmRNA can be delivered to the skin by several different approaches known in the art. Most topical delivery approaches have been shown to work for delivery of DNA, such as but not limited to, topical application of non-cationic liposome–DNA complex, cationic liposome–DNA complex, particle-mediated (gene gun), puncture-mediated gene transfections, and viral delivery approaches. After delivery of the nucleic acid, gene products have been detected in a number of different skin cell types, including, but not limited to, basal keratinocytes, sebaceous gland cells, dermal fibroblasts and dermal macrophages.

[00414] In one embodiment, the invention provides for a variety of dressings (e.g., wound dressings) or bandages (e.g., adhesive bandages) for conveniently and/or effectively carrying out methods of the present invention. Typically dressing or bandages may comprise sufficient amounts of pharmaceutical compositions and/or polynucleotides, primary constructs or mmRNA described herein to allow a user to perform multiple treatments of a subject(s).

[00415] In one embodiment, the invention provides for the polynucleotides, primary constructs or mmRNA compositions to be delivered in more than one injection.

[00416] In one embodiment, before topical and/or transdermal administration at least one area of tissue, such as skin, may be subjected to a device and/or solution which may increase permeability. In one embodiment, the tissue may be subjected to an abrasion device to increase the permeability of the skin (see U.S. Patent Publication No. 20080275468, herein incorporated by reference in its entirety). In another embodiment, the tissue may be subjected to an ultrasound enhancement device. An ultrasound enhancement device may include, but is not limited to, the devices described in U.S. Publication No. 20040236268 and U.S. Patent Nos. 6,491,657 and 6,234,990; each of which is herein incorporated by reference in their entireties. Methods of enhancing the permeability of tissue are described in U.S. Publication Nos. 20040171980 and 20040236268 and U.S. Pat. No. 6,190,315; each of whish are herein incorporated by reference in their entireties.

[00417] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of the polynucleotides, primary constructs and mmRNA described herein. The permeability of skin may be measured by methods known in the art and/or described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety. As a non-limiting example, a modified mRNA formulation may be delivered by the drug delivery methods described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[00418] In another non-limiting example tissue may be treated with a eutectic mixture of local anesthetics (EMLA) cream before, during and/or after the tissue may be subjected to a device which may increase permeability. Katz et al. (Anesth Analg (2004); 98:371-76; herein incorporated by reference in its entirety) showed that using the EMLA cream in combination with a low energy, an onset of superficial cutaneous analgesia was seen as fast as 5 minutes after a pretreatment with a low energy ultrasound.

[00419] In one embodiment, enhancers may be applied to the tissue before, during, and/or after the tissue has been treated to increase permeability. Enhancers include, but are not limited to, transport enhancers, physical enhancers, and cavitation enhancers. Non-limiting examples of enhancers are described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[00420] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of polynucleotides, primary constructs and/or mmRNA described herein, which may further contain a substance that invokes an immune response. In another non-limiting example, a formulation containing a substance to invoke an immune response may be delivered by the methods described in U.S. Publication Nos. 20040171980 and 20040236268; each of which is herein incorporated by reference in their entirety.

[00421] Dosage forms for topical and/or transdermal administration of a composition may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches. Generally, an active ingredient is admixed under sterile conditions with a pharmaceutically acceptable excipient and/or any needed preservatives and/or buffers as may be required.

[00422] Additionally, the present invention contemplates the use of transdermal patches, which often have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms may be prepared, for example, by dissolving and/or dispensing the compound in the proper medium. Alternatively or additionally, rate may be controlled by either providing a rate controlling membrane and/or by dispersing the compound in a polymer matrix and/or gel.

[00423] Formulations suitable for topical administration include, but are not limited to, liquid and/or semi liquid preparations such as liniments, lotions, oil in water and/or water in oil emulsions such as creams, ointments and/or pastes, and/or solutions and/or suspensions. Topically-administrable formulations may, for example, comprise from about 0.1% to about 10% (w/w) active ingredient, although the concentration of active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

Depot Administration

[00424] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a "target tissue") is targeted for administration.

[00425] In some aspects of the invention, the polynucleotides, primary constructs or mmRNA are spatially retained within or proximal to a target tissue. Provided are method of providing a composition to a target tissue of a mammalian subject by contacting the target tissue (which contains one or more target cells) with the composition under conditions such that the composition, in particular the nucleic acid component(s) of the composition, is substantially retained in the target tissue, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissue. Advantageously, retention is determined by measuring the amount of the nucleic acid present in the composition that enters one or more target cells. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the nucleic acids administered to the subject are present intracellularly at a period of time following administration. For example, intramuscular injection to a mammalian subject is performed using an aqueous composition containing a ribonucleic acid and a transfection reagent, and retention of the composition is determined by measuring the amount of the ribonucleic acid present in the muscle cells.

[00426] Aspects of the invention are directed to methods of providing a composition to a target tissue of a mammalian subject, by contacting the target tissue (containing one or more target cells) with the composition under conditions such that the composition is substantially retained in the target tissue. The composition contains an effective amount of a polynucleotide, primary construct or mmRNA such that the polypeptide of interest is produced in at least one target cell. The compositions generally contain a cell penetration agent, although "naked" nucleic acid (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[00427] In some circumstances, the amount of a protein produced by cells in a tissue is desirably increased. Preferably, this increase in protein production is spatially restricted to cells within the target tissue. Thus, provided are methods of increasing production of a protein of interest in a tissue of a mammalian subject. A composition is provided that contains polynucleotides, primary constructs or mmRNA

characterized in that a unit quantity of composition has been determined to produce the polypeptide of interest in a substantial percentage of cells contained within a predetermined volume of the target tissue.

[00428] In some embodiments, the composition includes a plurality of different polynucleotides, primary constructs or mmRNA, where one or more than one of the polynucleotides, primary constructs or mmRNA encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the composition. A determination is made of the dose of the composition required to produce the polypeptide of interest in a substantial percentage of cells contained within the predetermined volume of the target tissue (generally, without inducing significant production of the polypeptide of interest in tissue adjacent to the predetermined volume, or distally to the target tissue). Subsequent to this determination, the determined dose is introduced directly into the tissue of the mammalian subject.

[00429] In one embodiment, the invention provides for the polynucleotides, primary constructs or mmRNA to be delivered in more than one injection or by split dose injections.

[00430] In one embodiment, the invention may be retained near target tissue using a small disposable drug reservoir or patch pump. Non-limiting examples of patch pumps include those manufactured and/or sold by BD® (Franklin Lakes, NJ), Insulet Corporation (Bedford, MA), SteadyMed Therapeutics (San Francisco, CA), Medtronic (Minneapolis, MN), UniLife (York, PA), Valeritas (Bridgewater, NJ), and SpringLeaf Therapeutics (Boston, MA).

Pulmonary Administration

[00431] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 nm to about 7 nm or from about 1 nm to about 6 nm. Such compositions are suitably in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder and/or using a self propelling

solvent/powder dispensing container such as a device comprising the active ingredient dissolved and/or suspended in a low-boiling propellant in a sealed container. Such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nm and at least 95% of the particles by number have a diameter less than 7 nm. Alternatively, at least 95% of the particles by weight have a diameter greater than 1 nm and at least 90% of the particles by number have a diameter less than 6 nm. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

[00432] Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50% to 99.9% (w/w) of the composition, and active ingredient may constitute 0.1% to 20% (w/w) of the composition. A propellant may further comprise additional ingredients such as a liquid non-ionic and/or solid anionic surfactant and/or a solid diluent (which may have a particle size of the same order as particles comprising the active ingredient).

[00433] Pharmaceutical compositions formulated for pulmonary delivery may provide an active ingredient in the form of droplets of a solution and/or suspension. Such formulations may be prepared, packaged, and/or sold as aqueous and/or dilute alcoholic solutions and/or suspensions, optionally sterile, comprising active ingredient, and may conveniently be administered using any nebulization and/or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, and/or a preservative such as methylhydroxybenzoate. Droplets provided by this route of administration may have an average diameter in the range from about 0.1 nm to about 200 nm.

Intranasal, nasal and buccal Administration

[00434] Formulations described herein as being useful for pulmonary delivery are useful for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a

formulation is administered in the manner in which snuff is taken, *i.e.* by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[00435] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, 0.1% to 20% (w/w) active ingredient, the balance comprising an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

Ophthalmic Administration

[00436] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in an aqueous or oily liquid excipient. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form and/or in a liposomal preparation. Ear drops and/or eye drops are contemplated as being within the scope of this invention.

Payload Administration: Detectable Agents and Therapeutic Agents

[00437] The polynucleotides, primary constructs or mmRNA described herein can be used in a number of different scenarios in which delivery of a substance (the "payload") to a biological target is desired, for example delivery of detectable

substances for detection of the target, or delivery of a therapeutic agent. Detection methods can include, but are not limited to, both imaging *in vitro* and *in vivo* imaging methods, *e.g.*, immunohistochemistry, bioluminescence imaging (BLI), Magnetic Resonance Imaging (MRI), positron emission tomography (PET), electron microscopy, X-ray computed tomography, Raman imaging, optical coherence tomography, absorption imaging, thermal imaging, fluorescence reflectance imaging, fluorescence microscopy, fluorescence molecular tomographic imaging, nuclear magnetic resonance imaging, X-ray imaging, ultrasound imaging, photoacoustic imaging, lab assays, or in any situation where tagging/staining/imaging is required.

[00438] The polynucleotides, primary constructs or mmRNA can be designed to include both a linker and a payload in any useful orientation. For example, a linker having two ends is used to attach one end to the payload and the other end to the nucleobase, such as at the C-7 or C-8 positions of the deaza-adenosine or deaza-guanosine or to the N-3 or C-5 positions of cytosine or uracil. The polynucleotide of the invention can include more than one payload (e.g., a label and a transcription inhibitor), as well as a cleavable linker. In one embodiment, the modified nucleotide is a modified 7-deaza-adenosine triphosphate, where one end of a cleavable linker is attached to the C7 position of 7-deaza-adenine, the other end of the linker is attached to an inhibitor (e.g., to the C5 position of the nucleobase on a cytidine), and a label (e.g., Cy5) is attached to the center of the linker (see, e.g., compound 1 of A*pCp C5 Parg Capless in Fig. 5 and columns 9 and 10 of U.S. Pat. No. 7,994,304, incorporated herein by reference). Upon incorporation of the modified 7-deaza-adenosine triphosphate to an encoding region, the resulting polynucleotide will have a cleavable linker attached to a label and an inhibitor (e.g., a polymerase inhibitor). Upon cleavage of the linker (e.g., with reductive conditions to reduce a linker having a cleavable disulfide moiety), the label and inhibitor are released. Additional linkers and payloads (e.g., therapeutic agents, detectable labels, and cell penetrating payloads) are described herein.

[00439] For example, the polynucleotides, primary constructs or mmRNA described herein can be used in induced pluripotent stem cells (iPS cells), which can directly track cells that are transfected compared to total cells in the cluster. In another

example, a drug that may be attached to the polynucleotides, primary constructs or mmRNA via a linker and may be fluorescently labeled can be used to track the drug *in vivo*, *e.g.* intracellularly. Other examples include, but are not limited to, the use of a polynucleotide, primary construct or mmRNA in reversible drug delivery into cells.

[00440] The polynucleotides, primary constructs or mmRNA described herein can be used in intracellular targeting of a payload, *e.g.*, detectable or therapeutic agent, to specific organelle. Exemplary intracellular targets can include, but are not limited to, the nuclear localization for advanced mRNA processing, or a nuclear localization sequence (NLS) linked to the mRNA containing an inhibitor.

[00441] In addition, the polynucleotides, primary constructs or mmRNA described herein can be used to deliver therapeutic agents to cells or tissues, *e.g.*, in living animals. For example, the polynucleotides, primary constructs or mmRNA attached to the therapeutic agent through a linker can facilitate member permeation allowing the therapeutic agent to travel into a cell to reach an intracellular target.

[00442] In some embodiments, the payload may be a therapeutic agent such as a cytotoxin, radioactive ion, chemotherapeutic, or other therapeutic agent. A cytotoxin or cytotoxic agent includes any agent that may be detrimental to cells. Examples include, but are not limited to, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxyanthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, *e.g.*, maytansinol (see U.S. Pat. No. 5,208,020 incorporated herein in its entirety), rachelmycin (CC-1065, see U.S. Pat. Nos. 5,475,092, 5,585,499, and 5,846,545, all of which are incorporated herein by reference), and analogs or homologs thereof. Radioactive ions include, but are not limited to iodine (*e.g.*, iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90, samarium 153, and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thiotepa chlorambucil, rachelmycin (CC-1065), melphalan, carmustine (BSNU), lomustine

(CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, taxol and maytansinoids).

[00443] In some embodiments, the payload may be a detectable agent, such as various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aequorin), chemiluminescent materials, radioactive materials (e.g., ^{18}F, ^{67}Ga, ^{81m}Kr, ^{82}Rb, ^{111}In, ^{123}I, ^{133}Xe, ^{201}Tl, ^{125}I, ^{35}S, ^{14}C, ^{3}H, or ^{99m}Tc (e.g., as pertechnetate (technetate(VII), TcO_4^-)), and contrast agents (e.g., gold (*e.g.*, gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrystalline iron oxide nanoparticles (MIONs), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons). Such optically-detectable labels include for example, without limitation, 4-acetamido-4'-isothiocyanatostilbene-2,2′disulfonic acid; acridine and derivatives (e.g., acridine and acridine isothiocyanate); 5-(2′-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-l-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives (e.g., coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), and 7-amino-4-trifluoromethylcoumarin (Coumarin 151)); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5′ 5"-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2′-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2′-disulfonic acid; 5-[dimethylamino]-naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives (e.g., eosin and eosin isothiocyanate); erythrosin and derivatives (e.g., erythrosin B

and erythrosin isothiocyanate); ethidium; fluorescein and derivatives (e.g., 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2′,7'-dimethoxy-4'5′-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, X-rhodamine-5-(and-6)-isothiocyanate (QFITC or XRITC), and fluorescamine); 2-[2-[3-[[1,3-dihydro-1,1-dimethyl-3-(3-sulfopropyl)-2H-benz[e]indol-2-ylidene]ethylidene]-2-[4-(ethoxycarbonyl)-1-piperazinyl]-1-cyclopenten-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulforpropyl)-1H-benz[e]indolium hydroxide, inner salt, compound with n,n-diethylethanamine(1:1) (IR144); 5-chloro-2-[2-[3-[(5-chloro-3-ethyl-2(3H)-benzothiazol- ylidene)ethylidene]-2-(diphenylamino)-1-cyclopenten-1-yl]ethenyl]-3-ethyl benzothiazolium perchlorate (IR140); Malachite Green isothiocyanate; 4-methylumbelliferone orthocresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives(e.g., pyrene, pyrene butyrate, and succinimidyl 1-pyrene); butyrate quantum dots; Reactive Red 4 (CIBACRON™ Brilliant Red 3B-A); rhodamine and derivatives (e.g., 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodarnine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red), N,N,N',N'tetramethyl-6-carboxyrhodamine (TAMRA) tetramethyl rhodamine, and tetramethyl rhodamine isothiocyanate (TRITC)); riboflavin; rosolic acid; terbium chelate derivatives; Cyanine-3 (Cy3); Cyanine-5 (Cy5); cyanine-5.5 (Cy5.5), Cyanine-7 (Cy7); IRD 700; IRD 800; Alexa 647; La Jolta Blue; phthalo cyanine; and naphthalo cyanine.

[00444] In some embodiments, the detectable agent may be a non-detectable pre-cursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical))). *In vitro* assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis.

Combinations

[00445] The polynucleotides, primary constructs or mmRNA may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure encompasses the delivery of pharmaceutical, prophylactic, diagnostic, or imaging compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body. As a non-limiting example, the polynucleotides, primary constructs and/or mmRNA may be used in combination with a pharmaceutical agent for the treatment of cancer or to control hyperproliferative cells. In U.S. Pat. No. 7,964,571, herein incorporated by reference in its entirety, a combination therapy for the treatment of solid primary or metastasized tumor is described using a pharmaceutical composition including a DNA plasmid encoding for interleukin-12 with a lipopolymer and also administering at least one anticancer agent or chemotherapeutic. Further, the polynucleotides, primary constructs and/or mmRNA of the present invention that encodes anti-proliferative molecules may be in a pharmaceutical composition with a lipopolymer (see e.g., U.S. Pub. No. 20110218231, herein incorporated by reference in its entirety, claiming a pharmaceutical composition comprising a DNA plasmid encoding an anti-proliferative molecule and a lipopolymer) which may be administered with at least one chemotherapeutic or anticancer agent.

Dosing

[00446] The present invention provides methods comprising administering polynucleotides, primary constructs and/or mmRNA and their encoded proteins or complexes in accordance with the invention to a subject in need thereof. nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic

compositions thereof, may be administered to a subject using any amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition (*e.g.*, a disease, disorder, and/or condition relating to working memory deficits). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[00447] In certain embodiments, compositions in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect. The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage

may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations).

[00448] According to the present invention, it has been discovered that administration of mmRNA in split-dose regimens produce higher levels of proteins in mammalian subjects. As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses, e.g, two or more administrations of the single unit dose. As used herein, a "single unit dose" is a dose of any therapeutic administed in one dose/at one time/single route/single point of contact, i.e., single administration event. As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In one embodiment, the mmRNA of the present invention are administed to a subject in split doses. The mmRNA may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[00449] A pharmaceutical composition described herein can be formulated into a dosage form described herein, such as a topical, intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intracardiac, intraperitoneal, subcutaneous).

Liquid dosage forms

[00450] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art including, but not limited to, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. In certain embodiments for parenteral administration, compositions may be mixed with solubilizing agents such as CREMOPHOR®,

alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

Injectable

[00451] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art and may include suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include, but are not limited to, are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[00452] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00453] In order to prolong the effect of an active ingredient, it may be desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the polynucleotide, primary construct or mmRNA then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered polynucleotide, primary construct or mmRNA may be accomplished by dissolving or suspending the polynucleotide, primary construct or mmRNA in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the polynucleotide, primary construct or mmRNA in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of polynucleotide, primary construct or mmRNA to polymer and the nature of the particular polymer employed, the rate of

polynucleotide, primary construct or mmRNA release can be controlled. Examples of other biodegradable polymers include, but are not limited to, poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping the polynucleotide, primary construct or mmRNA in liposomes or microemulsions which are compatible with body tissues.

Pulmonary

[00454] Formulations described herein as being useful for pulmonary delivery may also be use for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration may be a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation may be administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[00455] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, contain about 0.1% to 20% (w/w) active ingredient, where the balance may comprise an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

[00456] General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The Science and

Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference).

Coatings or Shells

[00457] Solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Properties of Pharmaceutical Compositions

[00458] The pharmaceutical compositions described herein can be characterized by one or more of bioavailability, therapeutic window and/or volume of distribution.

Bioavailability

[00459] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in bioavailability as compared to a composition lacking a delivery agent as described herein. As used herein, the term "bioavailability" refers to the systemic availability of a given amount of polynucleotides, primary constructs or mmRNA administered to a mammal. Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration (C_{max}) of the unchanged form of a compound following administration of the compound to a mammal. AUC is a determination of the area under the curve plotting the serum or plasma concentration of a compound along the ordinate (Y-axis) against time along the abscissa (X-axis). Generally, the AUC for a particular compound can be calculated using methods known to those of ordinary skill in the art and as described in G. S. Banker, Modern Pharmaceutics, Drugs and the Pharmaceutical Sciences, v. 72, Marcel Dekker, New York, Inc., 1996, herein incorporated by reference.

[00460] The C_{max} value is the maximum concentration of the compound achieved in the serum or plasma of a mammal following administration of the compound to the mammal. The C_{max} value of a particular compound can be measured using methods known to those of ordinary skill in the art. The phrases "increasing bioavailability" or "improving the pharmacokinetics," as used herein mean that the systemic availability of a first polynucleotide, primary construct or mmRNA, measured as AUC, C_{max}, or C_{min} in a mammal is greater, when co-administered with a delivery agent as described herein, than when such co-administration does not take place. In some embodiments, the bioavailability of the polynucleotide, primary construct or mmRNA can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Therapeutic Window

[00461] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the administered polynucleotide, primary construct or mmRNA composition as compared to the therapeutic window of the administered polynucleotide, primary construct or mmRNA composition lacking a delivery agent as described herein. As used herein "therapeutic window" refers to the range of plasma concentrations, or the range of levels of therapeutically active substance at the site of action, with a high probability of eliciting a therapeutic effect. In some embodiments, the therapeutic window of the polynucleotide, primary construct or mmRNA when co-administered with a delivery agent as described herein can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Volume of Distribution

[00462] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution (V_{dist}), e.g., reduced or targeted, relative to a composition lacking a delivery agent as described herein. The volume of distribution (V_{dist}) relates the amount of the drug in the body to the concentration of the drug in the blood or plasma. As used herein, the term "volume of distribution" refers to the fluid volume that would be required to contain the total amount of the drug in the body at the same concentration as in the blood or plasma: V_{dist} equals the amount of drug in the body/concentration of drug in blood or plasma. For example, for a 10 mg dose and a plasma concentration of 10 mg/L, the volume of distribution would be 1 liter. The volume of distribution reflects the extent to which the drug is present in the extravascular tissue. A large volume of distribution reflects the tendency of a compound to bind to the tissue components compared with plasma protein binding. In a clinical setting, V_{dist} can be used to determine a loading dose to achieve a steady state concentration. In some embodiments, the volume of distribution of the polynucleotide, primary construct or mmRNA when co-administered with a delivery agent as described herein can decrease at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%.

Biological Effect

[00463] In one embodiment, the biological effect of the modified mRNA delivered to the animals may be categorized by analyzing the protein expression in the animals. The reprogrammed protein expression may be determined from analyzing a biological sample collected from a mammal administered the modified mRNA of the present invention. In one embodiment, the expression protein encoded by the modified mRNA administered to the mammal of at least 50 pg/ml may be preferred. For example, a protein expression of 50-200 pg/ml for the protein encoded by the modified mRNA delivered to the mammal may be seen as a therapeutically effective amount of protein in the mammal.

Detection of Modified Nucleic Acids by Mass Spectrometry

[00464] Mass spectrometry (MS) is an analytical technique that can provide structural and molecular mass/concentration information on molecules after their conversion to ions. The molecules are first ionized to acquire positive or negative charges and then they travel through the mass analyzer to arrive at different areas of the detector according to their mass/charge (m/z) ratio.

[00465] Mass spectrometry is performed using a mass spectrometer which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. For example ionization of the sample may be performed by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), photoionization, electron ionization, fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption/ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray ionization, and particle beam ionization. The skilled artisan will understand that the choice of ionization method can be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc.

[00466] After the sample has been ionized, the positively charged or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio (i.e., m/z). Suitable analyzers for determining mass-to-charge ratios include quadropole analyzers, ion traps analyzers, and time-of-flight analyzers. The ions may be detected using several detection modes. For example, selected ions may be detected (i.e., using a selective ion monitoring mode (SIM)), or alternatively, ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM).

[00467] Liquid chromatography-multiple reaction monitoring (LC-MS/MRM) coupled with stable isotope labeled dilution of peptide standards has been shown to be an effective method for protein verification (e.g., Keshishian et al., Mol Cell Proteomics 2009 8: 2339-2349; Kuhn et al., Clin Chem 2009 55:1108-1117; Lopez et al., Clin Chem 2010 56:281-290). Unlike untargeted mass spectrometry frequently used in biomarker discovery studies, targeted MS methods are peptide sequence–based modes of MS that focus the full analytical capacity of the instrument on tens to

hundreds of selected peptides in a complex mixture. By restricting detection and fragmentation to only those peptides derived from proteins of interest, sensitivity and reproducibility are improved dramatically compared to discovery-mode MS methods. This method of mass spectrometry-based multiple reaction monitoring (MRM) quantitation of proteins can dramatically impact the discovery and quantitation of biomarkers via rapid, targeted, multiplexed protein expression profiling of clinical samples.

[00468] In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be analyzed by the method of MRM-MS. The quantification of the biological sample may further include, but is not limited to, isotopically labeled peptides or proteins as internal standards.

[00469] According to the present invention, the biological sample, once obtained from the subject, may be subjected to enzyme digestion. As used herein, the term "digest" means to break apart into shorter peptides. As used herein, the phrase "treating a sample to digest proteins" means manipulating a sample in such a way as to break down proteins in a sample. These enzymes include, but are not limited to, trypsin, endoproteinase Glu-C and chymotrypsin. In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be digested using enzymes.

[00470] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein using electrospray ionization. Electrospray ionization (ESI) mass spectrometry (ESIMS) uses electrical energy to aid in the transfer of ions from the solution to the gaseous phase before they are analyzed by mass spectrometry. Samples may be analyzed using methods known in the art (e.g., Ho et al., Clin Biochem Rev. 2003 24(1):3-12). The ionic species contained in solution may be transferred into the gas phase by dispersing a fine spray of charge droplets, evaporating the solvent and ejecting the ions from the charged droplets to generate a mist of highly charged droplets. The mist of highly charged droplets may be analyzed using at least 1, at least 2, at least 3 or at least 4 mass analyzers such as, but not limited to, a quadropole mass analyzer.

Further, the mass spectrometry method may include a purification step. As a non-limiting example, the first quadrapole may be set to select a single m/z ratio so it may filter out other molecular ions having a different m/z ratio which may eliminate complicated and time-consuming sample purification procedures prior to MS analysis.

[00471] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein in a tandem ESIMS system (e.g., MS/MS). As non-limiting examples, the droplets may be analyzed using a product scan (or daughter scan) a precursor scan (parent scan) a neutral loss or a multiple reaction monitoring.

[00472] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MALDIMS). MALDI provides for the nondestructive vaporization and ionization of both large and small molecules, such as proteins. In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, which may also include, but is not limited to, an ultraviolet absorbing weak organic acid. Non-limiting examples of matrices used in MALDI are α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. Laser radiation of the analyte-matrix mixture may result in the vaporization of the matrix and the analyte. The laser induced desorption provides high ion yields of the intact analyte and allows for measurement of compounds with high accuracy. Samples may be analyzed using methods known in the art (e.g., Lewis, Wei and Siuzdak, Encyclopedia of Analytical Chemistry 2000:5880-5894). As non-limiting examples, mass analyzers used in the MALDI analysis may include a linear time-of-flight (TOF), a TOF reflectron or a Fourier transform mass analyzer.

[00473] In one embodiment, the analyte-matrix mixture may be formed using the dried-droplet method. A biologic sample is mixed with a matrix to create a saturated matrix solution where the matrix-to-sample ratio is approximately 5000:1. An aliquot (approximately 0.5-2.0 uL) of the saturated matrix solution is then allowed to dry to form the analyte-matrix mixture.

[00474] In one embodiment, the analyte-matrix mixture may be formed using the thin-layer method. A matrix homogeneous film is first formed and then the sample is then applied and may be absorbed by the matrix to form the analyte-matrix mixture.

[00475] In one embodiment, the analyte-matrix mixture may be formed using the thick-layer method. A matrix homogeneous film is formed with a nitro-cellulose matrix additive. Once the uniform nitro-cellulose matrix layer is obtained the sample is applied and absorbed into the matrix to form the analyte-matrix mixture.

[00476] In one embodiment, the analyte-matrix mixture may be formed using the sandwich method. A thin layer of matrix crystals is prepared as in the thin-layer method followed by the addition of droplets of aqueous trifluoroacetic acid, the sample and matrix. The sample is then absorbed into the matrix to form the analyte-matrix mixture.

V. Uses of Polynucleotides, primary constructs and mmRNA of the Invention

[00477] The polynucleotides, primary constructs and mmRNA of the present invention may be used to alter the phenotype of cells. The polynucleotides, primary constructs and mmRNA of the invention may encode peptides, polypeptides or multiple proteins to produce polypeptides of interest. The polypeptides of interest may be used in therapeutics and/or clinical and research settings. As a non-limiting example, the polypeptides of interest may include reprogramming factors, differentiation factors and de-differentiation factors.

Therapeutics

Therapeutic Agents

[00478] The polynucleotides, primary constructs or mmRNA of the present invention, such as modified nucleic acids and modified RNAs, and the proteins translated from them described herein can be used as therapeutic or prophylactic agents. They are provided for use in medicine, therapy and preventative treatments. For example, a polynucleotide, primary construct or mmRNA described herein can be administered to a subject, wherein the polynucleotide, primary construct or mmRNA is translated *in vivo* to produce a therapeutic or prophylactic polypeptide in the subject. Provided are compositions, methods, kits, and reagents for diagnosis, treatment or prevention of a disease or condition in humans and other mammals. The

active therapeutic agents of the invention include polynucleotides, primary constructs or mmRNA, cells containing the polynucleotides, primary constructs or mmRNA or polypeptides translated from the polynucleotides, primary constructs or mmRNA.

[00479] In certain embodiments, provided herein are combination therapeutics containing one or more polynucleotide, primary construct or mmRNA containing translatable regions that encode for a protein or proteins.

[00480] Provided herein are methods of inducing translation of a recombinant polypeptide in a cell population using the polynucleotide, primary construct or mmRNA described herein. Such translation can be *in vivo*, *ex vivo*, *in culture*, or *in vitro*. The cell population is contacted with an effective amount of a composition containing a nucleic acid that has at least one nucleoside modification, and a translatable region encoding the recombinant polypeptide. The population is contacted under conditions such that the nucleic acid is localized into one or more cells of the cell population and the recombinant polypeptide is translated in the cell from the nucleic acid.

[00481] An "effective amount" of the composition is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the nucleic acid (e.g., size, and extent of modified nucleosides), and other determinants. In general, an effective amount of the composition provides efficient protein production in the cell, preferably more efficient than a composition containing a corresponding unmodified nucleic acid. Increased efficiency may be demonstrated by increased cell transfection (i.e., the percentage of cells transfected with the nucleic acid), increased protein translation from the nucleic acid, decreased nucleic acid degradation (as demonstrated, e.g., by increased duration of protein translation from a modified nucleic acid), or reduced innate immune response of the host cell.

[00482] Aspects of the invention are directed to methods of inducing *in vivo* translation of a recombinant polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a nucleic acid that has at least one structural or chemical modification and a translatable region encoding the recombinant polypeptide is administered to the subject using the delivery methods described herein. The nucleic acid is provided in an amount and under other

conditions such that the nucleic acid is localized into a cell of the subject and the recombinant polypeptide is translated in the cell from the nucleic acid. The cell in which the nucleic acid is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of nucleic acid administration.

[00483] In certain embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that provide a functional activity which is substantially absent in the cell, tissue or organism in which the recombinant polypeptide is translated. For example, the missing functional activity may be enzymatic, structural, or gene regulatory in nature. In related embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that increases (e.g., synergistically) a functional activity which is present but substantially deficient in the cell in which the recombinant polypeptide is translated.

[00484] In other embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the recombinant polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or regulatory pathway thereof. In some embodiments, the recombinant polypeptide increases the level of an endogenous protein in the cell to a desirable level; such an increase may bring the level of the endogenous protein from a subnormal level to a normal level or from a normal level to a super-normal level.

[00485] Alternatively, the recombinant polypeptide functions to antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. Usually, the activity of the endogenous protein is deleterious to the subject; for example, due to mutation of the endogenous protein resulting in altered activity or localization. Additionally, the recombinant polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell. Examples of antagonized biological moieties include lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, a protein toxin such as shiga and tetanus toxins, or a small molecule

toxin such as botulinum, cholera, and diphtheria toxins. Additionally, the antagonized biological molecule may be an endogenous protein that exhibits an undesirable activity, such as a cytotoxic or cytostatic activity.

[00486] The recombinant proteins described herein may be engineered for localization within the cell, potentially within a specific compartment such as the nucleus, or are engineered for secretion from the cell or translocation to the plasma membrane of the cell.

[00487] Other aspects of the present disclosure relate to transplantation of cells containing polynucleotide, primary construct, or mmRNA to a mammalian subject. Administration of cells to mammalian subjects is known to those of ordinary skill in the art, and include, but is not limited to, local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), and the formulation of cells in pharmaceutically acceptable carrier. Such compositions containing polynucleotide, primary construct, or mmRNA can be formulated for administration intramuscularly, transarterially, intraperitoneally, intravenously, intranasally, subcutaneously, endoscopically, transdermally, or intrathecally. In some embodiments, the composition may be formulated for extended release.

The subject to whom the therapeutic agent may be administered suffers from or may be at risk of developing a disease, disorder, or deleterious condition. Provided are methods of identifying, diagnosing, and classifying subjects on these bases, which may include clinical diagnosis, biomarker levels, genome-wide association studies (GWAS), and other methods known in the art.

Diseases or Disorders

Familial Hypercholesterolemia

[00488] In one embodiment, the polynucleotide, primary construct, or mmRNA of the present invention may be used to treat familial hypercholesterolemia (FH). As used herein, the term "familial hypercholesterolemia" or "FH" refers to an autosomal dominant genetic disorder characterized by elevated levels of low density lipoprotein (LDL)-associated cholesterol in the plasma. Compared with LDL cholesterol levels in normal patients (e.g.,< 130mg/dL), levels in heterozygous and homozygous FH

patients often rise to 350-550 mg/dL and to > 600 mg/dL, respectively. Elevation in LDL cholesterol at these levels in patients or subjects with FH leads to cholesterol deposition within tisssues and may have an increased risk for cardiovascular disease at a young age. In some embodiments, high levels of LDL in the blood of these individuals may be the result of mutations in the gene encoding the LDL receptor. LDL is produced within the circulation by lipolytic catabolism of triglyeride-rich very low density lipoproteins or VLDL. Following lipid transfer and esterification reactions,it is believed, and is no means limiting, that the LDL receptor binds LDL in the circulation and facilitates endocytosis of LDL into the hepatic cell surface that the receptor is expressed on. When this receptor is dysfunctional, LDL levels remain elevated in the circulation during to their prolonged retention in the bloodstream and promote the development of atherosclerosis. Inactivating mutations in the LDLR gene are responsible for the majority of FH cases with LDLR expression in heterogygous and homozygous patients generally ~50% and ~10-15% of normal, respectively. Individuals with FH may be heterozygous or homozygous for FH-related gene mutations. Heterozygous FH is one of the most common genetic disorders with a prevelance of ~1/500 in the general population; while homozygous forms of the disease are more rare with a prevelance of ~1/1,000,000. Symptoms in homozygous individuals can be more severe. Diagnosis is possible during childhood or young adulthood by methods known in the art including, but not limited to, a physical exam that reveals xanthomas (fatty skin growths). Earlier diagnosis of FH may be made through an analysis of family history and genetics. (Sjouke, B. et al., *Familial hypercholesterolemia: present and future management*. Curr Cardiol Rep. 2011 Dec;13(6):527-36; Avis, H.J. et al., *A systematic review and meta-analysis of statin therapy in children with familial hypercholesterolemia*. Arterioscler Thromb Vasc Biol. 2007 Aug;27(8):1803-10; each of which are herein incorporated by reference in their entireties).

[00489] Current therapeutic agents, such as statins, Niacin and resins, can reduce serum cholesterol levels, either directly or indirectly, through induction of LDLR expression in the liver. While effective for many heterozygous FH patients, these approaches can be problematic. At least 25-30% of patients taking these drugs fail to

achieve their desired LDL cholesterol goals. These agents can be even less effective in treating homozygous FH primarily due to the low residual levels of functional LDLRs in the liver of these patients. Most of these patients are non-responsive to statins and in severe forms of disease, treatment is limited to LDL apheresis and liver transplantation. Current treatments are not always successful in lowering LDL-Cholesterol levels to target; therefore, new treatments are urgently needed.

[00490] In one embodiment, patients with FH may be administered a composition comprising at least one polynucleotide, primary construct or mmRNA of the present invention. The polynucleotide, primary construct or mmRNA may encode a peptide, protein or fragment thereof such as, but not limited to, low density lipoprotein receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin/kexin type 9 (PCSK9).

[00491] In one embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof of LDLR. In another embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof.

[00492] In one embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof of APOB. In another embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof. In one embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof of PCSK9. In another embodiment, FH may be treated by administering a composition of the present invention comprising at least one polynucleotide, primary construct or mmRNA encoding a peptide, protein or fragment thereof.

[00493] In another embodiment, it may be useful to optimize the expression of a specific polypeptide in a cell line or collection of cell lines of potential interest, particularly a polypeptide of interest such as a protein variant of a reference protein having a known activity. In one embodiment, provided is a method of optimizing expression of a polypeptide of interest in a target cell, by providing a plurality of target cell types, and independently contacting with each of the plurality of target cell types a modified mRNA encoding a polypeptide. Additionally, culture conditions may be altered to increase protein production efficiency. Subsequently, the presence and/or level of the polypeptide of interest in the plurality of target cell types is detected and/or quantitated, allowing for the optimization of a polypeptide of interest's expression by selection of an efficient target cell and cell culture conditions relating thereto. Such methods may be useful when the polypeptide of interest contains one or more post-translational modifications or has substantial tertiary structure, which often complicate efficient protein production.

[00494] Methods and compositions described herein may be used to produce proteins which are capable of attenuating or blocking the endogenous agonist biological response and/or antagonizing a receptor or signaling molecule in a mammalian subject. For example, IL-12 and IL-23 receptor signaling may be enhanced in chronic autoimmune disorders such as multiple sclerosis and inflammatory diseases such as rheumatoid arthritis, psoriasis, lupus erythematosus, ankylosing spondylitis and Chron's disease (Kikly K, Liu L, Na S, Sedgwich JD (2006) Cur. Opin. Immunol. 18(6): 670-5). In another embodiment, a nucleic acid encodes an antagonist for chemokine receptors. Chemokine receptors CXCR-4 and CCR-5 are required for HIV enry into host cells (Arenzana-Seisdedos F et al, (1996) Nature. Oct 3; 383 (6599):400).

Expression of Ligand or Receptor on Cell Surface

[00495] In some aspects and embodiments of the aspects described herein, the polynucleotides, primary constructs or mmRNA described herein can be used to express a ligand or ligand receptor on the surface of a cell *(e.g.,* a homing moiety). A ligand or ligand receptor moiety attached to a cell surface can permit the cell to have a desired biological interaction with a tissue or an agent *in vivo.* A ligand can be an

antibody, an antibody fragment, an aptamer, a peptide, a vitamin, a carbohydrate, a protein or polypeptide, a receptor, *e.g.*, cell-surface receptor, an adhesion molecule, a glycoprotein, a sugar residue, a therapeutic agent, a drug, a glycosaminoglycan, or any combination thereof. For example, a ligand can be an antibody that recognizes a cancer-cell specific antigen, rendering the cell capable of preferentially interacting with tumor cells to permit tumor-specific localization of a modified cell. A ligand can confer the ability of a cell composition to accumulate in a tissue to be treated, since a preferred ligand may be capable of interacting with a target molecule on the external face of a tissue to be treated. Ligands having limited cross-reactivity to other tissues are generally preferred.

[00496] In some cases, a ligand can act as a homing moiety which permits the cell to target to a specific tissue or interact with a specific ligand. Such homing moieties can include, but are not limited to, any member of a specific binding pair, antibodies, monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent binding reagents including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ((SCFV)2 fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized *(i.e.,* leucine zipper or helix stabilized) scFv fragments; and other homing moieties include for example, aptamers, receptors, and fusion proteins.

[00497] In some embodiments, the homing moiety may be a surface-bound antibody, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In one embodiment, multiple antibodies are expressed on the surface of a cell, and each antibody can have a different specificity for a desired target. Such approaches can increase the avidity and specificity of homing interactions.

[00498] A skilled artisan can select any homing moiety based on the desired localization or function of the cell, for example an estrogen receptor ligand, such as

tamoxifen, can target cells to estrogen-dependent breast cancer cells that have an increased number of estrogen receptors on the cell surface. Other non-limiting examples of ligand/receptor interactions include CCRI *(e.g.,* for treatment of inflamed joint tissues or brain in rheumatoid arthritis, and/or multiple sclerosis), CCR7, CCR8 *(e.g.,* targeting to lymph node tissue), CCR6, CCR9,CCR10 *(e.g.,* to target to intestinal tissue), CCR4, CCR10 *(e.g.,* for targeting to skin), CXCR4 *(e.g.,* for general enhanced transmigration), HCELL *(e.g.,* for treatment of inflammation and inflammatory disorders, bone marrow), Alpha4beta7 *(e.g.,* for intestinal mucosa targeting), VLA-4/VCAM-1 *(e.g.,* targeting to endothelium). In general, any receptor involved in targeting *(e.g.,* cancer metastasis) can be harnessed for use in the methods and compositions described herein.

VI. Kits and Devices

[00499] The invention provides a variety of kits for conveniently and/or effectively carrying out methods of the present invention. Typically kits will comprise sufficient amounts and/or numbers of components to allow a user to perform multiple treatments of a subject(s) and/or to perform multiple experiments, and contact cells and/or a population of cells at least once.

[00500] In one aspect, the present invention provides kits comprising the molecules (polynucleotides, primary constructs or mmRNA) of the invention. In one embodiment, the kit comprises one or more functional antibodies or function fragments thereof.

[00501] Kits and devices useful in combination with the polynucleotides, primary constructs or mmRNA) of the invention include those disclosed in co-pending U.S. Provisional Patent Application No 61/737,130 filed December 14, 2012, the contents of which are incorporated herein by reference in their entirety.

VII. Definitions

[00502] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual subcombination of the members of such groups and ranges. For example, the term "C_{1-6} alkyl" is

specifically intended to individually disclose methyl, ethyl, C_3 alkyl, C_4 alkyl, C_5 alkyl, and C_6 alkyl.

[00503] *About:* As used herein, the term "about" means +/- 10% of the recited value.

[00504] *Administered in combination:* As used herein, the term "administered in combination" or "combined administration" means that two or more agents are administered to a subject at the same time or within an interval such that there may be an overlap of an effect of each agent on the patient. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently closely together such that a combinatorial (*e.g.*, a synergistic) effect is achieved.

[00505] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans at any stage of development. In some embodiments, "animal" refers to non-human animals at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and worms. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

[00506] *Approximately:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[00507] *Associated with:* As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to

form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, *e.g.*, physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

[00508] *Bifunctional:* As used herein, the term "bifunctional" refers to any substance, molecule or moiety which is capable of or maintains at least two functions. The functions may effect the same outcome or a different outcome. The structure that produces the function may be the same or different. For example, bifunctional modified RNAs of the present invention may encode a cytotoxic peptide (a first function) while those nucleosides which comprise the encoding RNA are, in and of themselves, cytotoxic (second function). In this example, delivery of the bifunctional modified RNA to a cancer cell would produce not only a peptide or protein molecule which may ameliorate or treat the cancer but would also deliver a cytotoxic payload of nucleosides to the cell should degradation, instead of translation of the modified RNA, occur.

[00509] *Biocompatible*: As used herein, the term "biocompatible" means compatible with living cells, tissues, organs or systems posing little to no risk of injury, toxicity or rejection by the immune system.

[00510] *Biodegradable*: As used herein, the term "biodegradable" means capable of being broken down into innocuous products by the action of living things.

[00511] *Biologically active*: As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system and/or organism. For instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In particular embodiments, a polynucleotide, primary construct or mmRNA of the present invention may be considered biologically active if even a portion of the polynucleotide, primary construct or mmRNA is biologically active or mimics an activity considered biologically relevant.

[00512] *Cancer stem cells*: As used herein, "cancer stem cells" are cells that can undergo self-renewal and/or abnormal proliferation and differentiation to form a tumor.

[00513] *Chemical terms*: Chemical terms not otherwise defined herein, will conform to the chemical term definitions provided in co-pending U.S. Provisional Patent Application No 61/737,130 filed December 14, 2012, the contents of which are incorporated herein by reference in their entirety.

[00514] The term "diastereomer," as used herein means stereoisomers that are not mirror images of one another and are non-superimposable on one another.

[00515] The term "effective amount" of an agent, as used herein, is that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. For example, in the context of administering an agent that treats cancer, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of cancer, as compared to the response obtained without administration of the agent.

[00516] The term "enantiomer," as used herein, means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[00517] The term "isomer," as used herein, means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, e.g., racemates. Enantiomeric and

stereoisomeric mixtures of compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[00518] The term "stereoisomer," as used herein, refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[00519] *Compound:* As used herein, the term "compound," is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[00520] The compounds described herein can be asymmetric (*e.g.*, having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically active starting materials are known in the art, such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present disclosure. Cis and trans geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[00521] Compounds of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same

empirical formula and total charge. Examples prototropic tautomers include ketone – enol pairs, amide – imidic acid pairs, lactam – lactim pairs, amide – imidic acid pairs, enamine – imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1H- and 3H-imidazole, 1H-, 2H- and 4H-1,2,4-triazole, 1H- and 2H- isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[00522] Compounds of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. "Isotopes" refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[00523] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[00524] *Committed:* As used herein, the term "committed" means, when referring to a cell, when the cell is far enough into the differentiation pathway where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell type instead of into a different cell type or reverting to a lesser differentiated cell type.

[00525] *Conserved*: As used herein, the term "conserved" refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared. Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[00526] In some embodiments, two or more sequences are said to be "completely conserved" if they are 100% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are about 70% identical, about 80% identical, about 90% identical, about 95%, about

98%, or about 99% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are at least 30% identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of an oligonucleotide or polypeptide or may apply to a portion, region or feature thereof.

[00527] *Controlled Release:* As used herein, the term "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome.

[00528] *Cyclic or Cyclized:* As used herein, the term "cyclic" refers to the presence of a continuous loop. Cyclic molecules need not be circular, only joined to form an unbroken chain of subunits. Cyclic molecules such as the engineered RNA or mRNA of the present invention may be single units or multimers or comprise one or more components of a complex or higher order structure.

[00529] *Cytostatic*: As used herein, "cytostatic" refers to inhibiting, reducing, suppressing the growth, division, or multiplication of a cell (*e.g.*, a mammalian cell (*e.g.*, a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[00530] *Cytotoxic*: As used herein, "cytotoxic" refers to killing or causing injurious, toxic, or deadly effect on a cell (*e.g.*, a mammalian cell (*e.g.*, a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[00531] *Delivery:* As used herein, "delivery" refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[00532] *Delivery Agent*: As used herein, "delivery agent" refers to any substance which facilitates, at least in part, the *in vivo* delivery of a polynucleotide, primary construct or mmRNA to targeted cells.

[00533] *Destabilized:* As used herein, the term "destable," "destabilize," or "destabilizing region" means a region or molecule that is less stable than a starting, wild-type or native form of the same region or molecule.

[00534] *Detectable label:* As used herein, "detectable label" refers to one or more markers, signals, or moieties which are attached, incorporated or associated with another entity that is readily detected by methods known in the art including radiography, fluorescence, chemiluminescence, enzymatic activity, absorbance and the like. Detectable labels include radioisotopes, fluorophores, chromophores, enzymes, dyes, metal ions, ligands such as biotin, avidin, streptavidin and haptens, quantum dots, and the like. Detectable labels may be located at any position in the peptides or proteins disclosed herein. They may be within the amino acids, the peptides, or proteins, or located at the N- or C- termini.

[00535] *Developmental Potential*: As used herein, "developmental potential" or "developmental potency" refers to the total of all developmental cell fates or cell types that can be achieved by a cell upon differentiation.

[00536] *Developmental Potential Altering Factor*: As used herein, "developmental potential altering factor" refers to a protein or RNA which can alter the developmental potential of a cell.

[00537] *Digest*: As used herein, the term "digest" means to break apart into smaller pieces or components. When referring to polypeptides or proteins, digestion results in the production of peptides.

[00538] *Differentiated cell*: As used herein, the term "differentiated cell" refers to any somatic cell that is not, in its native form, pluripotent. Differentiated cell also encompasses cells that are partially differentiated.

[00539] *Differentiation*: As used herein, the term "differentiation factor" refers to a developmental potential altering factor such as a protein, RNA or small molecule that can induce a cell to differentiate to a desired cell-type.

[00540] *Differentiate*: As used herein, "differentiate" refers to the process where an uncommitted or less committed cell acquires the features of a committed cell.

[00541] *Distal:* As used herein, the term "distal" means situated away from the center or away from a point or region of interest.

[00542] *Dose splitting factor (DSF)*-ratio of PUD of dose split treatment divided by PUD of total daily dose or single unit dose. The value is derived from comparison of dosing regimens groups.

[00543] *Embryonic stem cell:* As used herein, the term "embryonic stem cell" refers to naturally occurring pluripotent stem cells of the inner cell mass of the embryonic blastocyst.

[00544] *Encapsulate:* As used herein, the term "encapsulate" means to enclose, surround or encase.

[00545] *Encoded protein cleavage signal*: As used herein, "encoded protein cleavage signal" refers to the nucleotide sequence which encodes a protein cleavage signal.

[00546] *Engineered:* As used herein, embodiments of the invention are "engineered" when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[00547] *Exosome:* As used herein, "exosome" is a vesicle secreted by mammalian cells or a complex involved in RNA degradation.

[00548] *Expression*: As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5′ cap formation, and/or 3′ end processing); (3) translation of an RNA into a polypeptide or protein; and (4) post-translational modification of a polypeptide or protein.

[00549] *Feature:* As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

[00550] *Formulation*: As used herein, a "formulation" includes at least a polynucleotide, primary construct or mmRNA and a delivery agent.

[00551] *Fragment:* A "fragment," as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

[00552] *Functional*: As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[00553] *Homology*: As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.* between nucleic acid molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). In accordance with the invention, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least about 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. In accordance with the invention, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids.

[00554] *Identity*: As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between oligonucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The

percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., *et al.*, *Nucleic Acids Research*, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. *et al.*, *J. Molec. Biol.*, 215, 403 (1990)).

[00555] *Inhibit expression of a gene:* As used herein, the phrase "inhibit expression of a gene" means to cause a reduction in the amount of an expression product of the gene. The expression product can be an RNA transcribed from the gene (*e.g.*, an mRNA) or a polypeptide translated from an mRNA transcribed from the gene.

Typically a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[00556] *In vitro*: As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, in a Petri dish, *etc.*, rather than within an organism (*e.g.*, animal, plant, or microbe).

[00557] *In vivo*: As used herein, the term "*in vivo*" refers to events that occur within an organism (*e.g.*, animal, plant, or microbe or cell or tissue thereof).

[00558] *Isolated*: As used herein, the term "isolated" refers to a substance or entity that has been separated from at least some of the components with which it was associated (whether in nature or in an experimental setting). Isolated substances may have varying levels of purity in reference to the substances from which they have been associated. Isolated substances and/or entities may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated agents are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components.

[00559] *Substantially isolated*: By "substantially isolated" is meant that the compound is substantially separated from the environment in which it was formed or detected. Partial separation can include, for example, a composition enriched in the compound of the present disclosure. Substantial separation can include compositions containing at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% by weight of the compound of the present disclosure, or salt thereof. Methods for isolating compounds and their salts are routine in the art.

[00560] *Linker:* As used herein, a linker refers to a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. The linker can be attached to a modified nucleoside or nucleotide on the nucleobase

or sugar moiety at a first end, and to a payload, e.g., a detectable or therapeutic agent, at a second end. The linker may be of sufficient length as to not interfere with incorporation into a nucleic acid sequence. The linker can be used for any useful purpose, such as to form mmRNA multimers (e.g., through linkage of two or more polynucleotides, primary constructs, or mmRNA molecules) or mmRNA conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers, Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bond include an amido bond can be cleaved for example by the use of tris(2-carboxyethyl)phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond can be cleaved for example by acidic or basic hydrolysis.

[00561] *MicroRNA (miRNA) binding site:* As used herein, a microRNA (miRNA) binding site represents a nucleotide location or region of a nucleic acid transcript to which at least the "seed" region of a miRNA binds.

[00562] *Modified:* As used herein "modified" refers to a changed state or structure of a molecule of the invention. Molecules may be modified in many ways including chemically, structurally, and functionally. In one embodiment, the mRNA molecules of the present invention are modified by the introduction of non-natural nucleosides and/or nucleotides, e.g., as it relates to the natural ribonucleotides A, U, G, and C. Noncanonical nucleotides such as the cap structures are not considered "modified" although they differ from the chemical structure of the A, C, G, U ribonucleotides.

[00563] *Mucus*: As used herein, "mucus" refers to the natural substance that is viscous and comprises mucin glycoproteins.

[00564] *Multipotent*: As used herein, "multipotent" or "partially differentiated cell" when referring to a cell refers to a cell that has a developmental potential to differentiate into cells of one or more germ layers, but not all three germ layers.

[00565] *Naturally occurring:* As used herein, "naturally occurring" means existing in nature without artificial aid.

[00566] *Non-human vertebrate:* As used herein, a "non human vertebrate" includes all vertebrates except *Homo sapiens*, including wild and domesticated species. Examples of non-human vertebrates include, but are not limited to, mammals, such as alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, gayal, goat, guinea pig, horse, llama, mule, pig, rabbit, reindeer, sheep water buffalo, and yak.

[00567] *Off-target:* As used herein, "off target" refers to any unintended effect on any one or more target, gene, or cellular transcript.

[00568] *Oligopotent:* As used herein, "oligopotent" when referring to a cell means to give rise to a more restricted subset of cell lineages than multipotent stem cells..

[00569] *Open reading frame:* As used herein, "open reading frame" or "ORF" refers to a sequence which does not contain a stop codon in a given reading frame.

[00570] *Operably linked:* As used herein, the phrase "operably linked" refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[00571] *Optionally substituted:* Herein a phrase of the form "optionally substituted X" (*e.g.*, optionally substituted alkyl) is intended to be equivalent to "X, wherein X is optionally substituted" (*e.g.*, "alkyl, wherein said alkyl is optionally substituted"). It is not intended to mean that the feature "X" (*e.g.* alkyl) *per se* is optional.

[00572] *Peptide:* As used herein, "peptide" is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[00573] *Paratope:* As used herein, a "paratope" refers to the antigen-binding site of an antibody.

[00574] *Patient:* As used herein, "patient" refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.

[00575] *Pharmaceutically acceptable*: The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00576] *Pharmaceutically acceptable excipients:* The phrase "pharmaceutically acceptable excipient," as used herein, refers any ingredient other than the compounds described herein (for example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspensing or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[00577] *Pharmaceutically acceptable salts*: The present disclosure also includes pharmaceutically acceptable salts of the compounds described herein. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not

limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, *Pharmaceutical Salts: Properties, Selection, and Use*, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., *Journal of Pharmaceutical Science*, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

[00578] *Pharmacokinetic:* As used herein, "pharmacokinetic" refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[00579] *Pharmaceutically acceptable solvate*: The term "pharmaceutically acceptable solvate," as used herein, means a compound of the invention wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), *N*-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), *N,N'*-dimethylformamide (DMF), *N,N'*-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate."

[00580] *Physicochemical:* As used herein, "physicochemical" means of or relating to a physical and/or chemical property.

[00581] *Pluripotent:* As used herein, "pluripotent" refers to a cell with the developmental potential, under different conditions, to differentiate to cell types characteristic of all three germ layers.

[00582] *Pluripotency:* As used herein, "pluripotency" or "pluripotent state" refers to the developmental potential of a cell where the cell has the ability to differentitate into all three embryonic germ layers (endoderm, mesoderm and ectoderm).

[00583] *Preventing*: As used herein, the term "preventing" refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[00584] *Prodrug*: The present disclosure also includes prodrugs of the compounds described herein. As used herein, "prodrugs" refer to any substance, molecule or entity which is in a form predicate for that substance, molecule or entity to act as a therapeutic upon chemical or physical alteration. Prodrugs may by covalently bonded or sequestered in some way and which release or are converted into the active drug moiety prior to, upon or after administered to a mammalian subject. Prodrugs can be prepared by modifying functional groups present in the compounds in such a way that the modifications are cleaved, either in routine manipulation or *in vivo*, to the parent compounds. Prodrugs include compounds wherein hydroxyl, amino, sulfhydryl, or carboxyl groups are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, amino, sulfhydryl, or carboxyl group respectively. Preparation and use of prodrugs is discussed in T. Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design*, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference in their entirety.

[00585] *Proliferate:* As used herein, the term "proliferate" means to grow, expand or increase or cause to grow, expand or increase rapidly. "Proliferative" means having

the ability to proliferate. "Anti-proliferative" means having properties counter to or inapposite to proliferative properties.

[00586] *Progenitor cell*: As used herein, the term "progenitor cell" refers to cells that have greater developmental potential relative to a cell which it can give rise to by differentiation.

[00587] *Protein cleavage site*: As used herein, "protein cleavage site" refers to a site where controlled cleavage of the amino acid chain can be accomplished by chemical, enzymatic or photochemical means.

[00588] *Protein cleavage signal:* As used herein "protein cleavage signal" refers to at least one amino acid that flags or marks a polypeptide for cleavage.

[00589] *Protein of interest:* As used herein, the terms "proteins of interest" or "desired proteins" include those provided herein and fragments, mutants, variants, and alterations thereof.

[00590] *Proximal:* As used herein, the term "proximal" means situated nearer to the center or to a point or region of interest.

[00591] *Purified:* As used herein, "purify," "purified," "purification" means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection.

[00592] *Repeated transfection:* As used herein, the term "repeated transfection" refers to transfection of the same cell culture with a polynucleotide, primary construct or mmRNA a plurality of times. The cell culture can be transfected at least twice, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 11 times, at least 12 times, at least 13 times, at least 14 times, at least 15 times, at least 16 times, at least 17 times at least 18 times, at least 19 times, at least 20 times, at least 25 times, at least 30 times, at least 35 times, at least 40 times, at least 45 times, at least 50 times or more.

[00593] *Reprogramming:* As used herein, "reprogramming" refers to a process that reverses the developmental potential of a cell or population of cells.

[00594] *Reprogramming factor:* As used herein, the term "reprogramming factor" refers to a developmental potential altering factor such as a protein, RNA or small

molecule, the expression of which contributes to the reprogramming of a cell to a less differentiated or undifferentiated state.

[00595] *Sample:* As used herein, the term "sample" or "biological sample" refers to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). A sample further may include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[00596] *Signal Sequences:* As used herein, the phrase "signal sequences" refers to a sequence which can direct the transport or localization of a protein.

[00597] *Single unit dose*: As used herein, a "single unit dose" is a dose of any therapeutic administed in one dose/at one time/single route/single point of contact, i.e., single administration event.

[00598] *Similarity*: As used herein, the term "similarity" refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art.

[00599] *Somatic cell*: As used herein, "somatic cells" refers to any cell other than a germ cell, a cell present in or obtained from a pre-implantation embryo, or a cell resulting from proliferation of such a cell *in vitro*.

[00600] *Somatic stem cell*: As used herein, a "somatic stem cell" refers to any pluripotent or multipotent stem cell derived from non-embryonic tissue including fetal, juvenile and adult tissue.

[00601] *Somatic pluripotent cell*: As used herein, a "somatic pluripotent cell" refers to a somatic cell that has had its developmental potential altered to that of a pluripotent state.

[00602] *Split dose*: As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses.

[00603] *Stable:* As used herein "stable" refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[00604] *Stabilized:* As used herein, the term "stabilize", "stabilized," "stabilized region" means to make or become stable.

[00605] *Stem cell:* As used herein, the term "stem cell" refers to a cell in an undifferentiated or partially differentiated state that has the property of self-renewal and ahs the developmental potential to differentiate into multiple cell types, without a specific developmental potential. A stem cell may be able capable of proliferation and giving rise to more such stem cells while maintaining its developmental potential.

[00606] *Subject:* As used herein, the term "subject" or "patient" refers to any organism to which a composition in accordance with the invention may be administered, *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[00607] *Substantially*: As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[00608] *Substantially equal*: As used herein as it relates to time differences between doses, the term means plus/minus 2%.

[00609] *Substantially simultaneously*: As used herein and as it relates to plurality of doses, the term means within 2 seconds.

[00610] *Suffering from*: An individual who is "suffering from" a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[00611] *Susceptible to*: An individual who is "susceptible to" a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[00612] *Sustained release:* As used herein, the term "sustained release" refers to a pharmaceutical composition or compound release profile that conforms to a release rate over a specific period of time.

[00613] *Synthetic*: The term "synthetic" means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present invention may be chemical or enzymatic.

[00614] *Targeted Cells:* As used herein, "targeted cells" refers to any one or more cells of interest. The cells may be found *in vitro*, *in vivo*, *in situ* or in the tissue or organ of an organism. The organism may be an animal, preferably a mammal, more preferably a human and most preferably a patient.

[00615] *Therapeutic Agent:* The term "therapeutic agent" refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[00616] *Therapeutically effective amount:* As used herein, the term "therapeutically effective amount" means an amount of an agent to be delivered (*e.g.*, nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, *etc.*) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[00617] *Therapeutically effective outcome*: As used herein, the term "therapeutically effective outcome" means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[00618] *Total daily dose:* As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose.

[00619] *Totipotency*: As used herein, "totipotency" refers to a cell with a developmental potential to make all of the cells found in the adult body as well as the extra-embryonic tissues, including the placenta.

[00620] *Transcription factor:* As used herein, the term "transcription factor" refers to a DNA-binding protein that regulates transcription of DNA into RNA, for example, by activation or repression of transcription. Some transcription factors effect regulation of transcription alone, while others act in concert with other proteins. Some transcription factor can both activate and repress transcription under certain conditions. In general, transcription factors bind a specific target sequence or sequences highly similar to a specific consensus sequence in a regulatory region of a target gene. Transcription factors may regulate transcription of a target gene alone or in a complex with other molecules.

[00621] *Transcription*: As used herein, the term "transcription" refers to methods to introduce exogenous nucleic acids into a cell. Methods of transfection include, but

are not limited to, chemical methods, plysical treatments and cationic lipids or mixtures.

[00622] *Transdifferentiation*: As used herein, "transdifferentiation" refers to the capacity of differentiated cells of one type to lose identifying characteristics and to change their phenotype to that of other fully differentiated cells.

[00623] *Treating*: As used herein, the term "treating" refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, "treating" cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[00624] *Unmodified*: As used herein, "unmodified" refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the "unmodified" starting molecule for a subsequent modification.

Equivalents and Scope

[00625] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[00626] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to

the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[00627] It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed.

[00628] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[00629] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (*e.g.*, any nucleic acid or protein encoded thereby; any method of production; any method of use; *etc.*) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[00630] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[00631] Section and table headings are not intended to be limiting.

EXAMPLES

Example 1. Modified mRNA Production

[00632] Modified mRNAs (mmRNA) according to the invention may be made using standard laboratory methods and materials. The open reading frame (ORF) of the gene of interest may be flanked by a 5' untranslated region (UTR) which may contain a strong Kozak translational initiation signal and/or an alpha-globin 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. The modified mRNAs may be modified to reduce the cellular innate immune response. The modifications to reduce the cellular response may include pseudouridine (ψ) and 5-methyl-cytidine (5meC, 5mc or m^5C). (See, Kariko K et al. Immunity 23:165-75 (2005), Kariko K et al. Mol Ther 16:1833-40 (2008), Anderson BR et al. NAR (2010); each of which is herein incorporated by reference in their entirety).

[00633] The ORF may also include various upstream or downstream additions (such as, but not limited to, β-globin, tags, etc.) may be ordered from an optimization service such as, but limited to, DNA2.0 (Menlo Park, CA) and may contain multiple cloning sites which may have XbaI recognition. Upon receipt of the construct, it may be reconstituted and transformed into chemically competent *E. coli*.

[00634] For the present invention, NEB DH5-alpha Competent *E. coli* are used. Transformations are performed according to NEB instructions using 100 ng of plasmid. The protocol is as follows:

1. Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes.
2. Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 µl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting.

[00635] Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

[00636] A single colony is then used to inoculate 5 ml of LB growth media using the appropriate antibiotic and then allowed to grow (250 RPM, 37° C) for 5 hours. This is

then used to inoculate a 200 ml culture medium and allowed to grow overnight under the same conditions.

[00637] To isolate the plasmid (up to 850 μg), a maxi prep is performed using the Invitrogen PURELINK™ HiPure Maxiprep Kit (Carlsbad, CA), following the manufacturer's instructions.

[00638] In order to generate cDNA for *In Vitro* Transcription (IVT), the plasmid is first linearized using a restriction enzyme such as XbaI. A typical restriction digest with XbaI will comprise the following: Plasmid 1.0 μg; 10x Buffer 1.0 μl; XbaI 1.5 μl; dH_20 up to 10 μl; incubated at 37° C for 1 hr. If performing at lab scale (< 5μg), the reaction is cleaned up using Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer's instructions. Larger scale purifications may need to be done with a product that has a larger load capacity such as Invitrogen's standard PURELINK™ PCR Kit (Carlsbad, CA). Following the cleanup, the linearized vector is quantified using the NanoDrop and analyzed to confirm linearization using agarose gel electrophoresis.

[00639] As a non-limiting example, G-CSF may represent the polypeptide of interest. Sequences used in the steps outlined in Examples 1-5 are shown in Table 3. It should be noted that the start codon (ATG) has been underlined in each sequence of Table 3.

Table 3. G-CSF Sequences

SEQ ID NO	Description
27	cDNAsequence: ATGGCTGGACCTGCCACCCAGAGCCCCATGAAGCTGATGGCCC TGCAGCTGCTGCTGTGGCACAGTGCACTCTGGACAGTGCAGGA AGCCACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTC CTGCTCAAGTGCTTAGAGCAAGTGAGGAAGATCCAGGGCGAT GGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCTACAAGCTGT GCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCAT CCCCTGGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAG CTGGCAGGCTGCTTGAGCCAACTCCATAGCGGCCTTTTCCTCTA CCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTG GGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTG CCACCACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCC CTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTCGCCTC TGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTTGCCTCCCAT

	CTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACC TTGCCCAGCCCTGA
28	cDNA having T7 polymerase site, AfeI and Xba restriction site: TAATACGACTCACTATA GGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGA GCCACC ATGGCTGGACCTGCCACCCAGAGCCCCATGAAGCTGATGGCCC TGCAGCTGCTGCTGTGGCACAGTGCACTCTGGACAGTGCAGGA AGCCACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTC CTGCTCAAGTGCTTAGAGCAAGTGAGGAAGATCCAGGGCGAT GGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCTACAAGCTGT GCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCAT CCCCTGGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAG CTGGCAGGCTGCTTGAGCCAACTCCATAGCGGCCTTTTCCTCTA CCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTG GGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTG CCACCACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCC CTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTCGCCTC TGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTTGCCTCCCAT CTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACC TTGCCCAGCCCTGA AGCGCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTT CTCTCCCTTGCACCTGTACCTCTTGGTCTTTGAATAAAGCCTGA GTAGGAAGGCGGCCGCTCGAGCATGCATCTAGA
29	Optimized sequence; containing T7 polymerase site, AfeI and Xba restriction site TAATACGACTCACTATA GGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGA GCCACC ATGGCCGGTCCCGCGACCCAAAGCCCCATGAAACTTATGGCCC TGCAGTTGCTGCTTTGGCACTCGGCCCTCTGGACAGTCCAAGA AGCGACTCCTCTCGGACCTGCCTCATCGTTGCCGCAGTCATTCC TTTTGAAGTGTCTGGAGCAGGTGCGAAAGATTCAGGGCGATGG AGCCGCACTCCAAGAGAAGCTCTGCGCGACATACAAACTTTGC CATCCCGAGGAGCTCGTACTGCTCGGGCACAGCTTGGGGATTC CCTGGGCTCCTCTCTCGTCCTGTCCGTCGCAGGCTTTGCAGTTG GCAGGGTGCCTTTCCCAGCTCCACTCCGGTTTGTTCTTGTATCA GGGACTGCTGCAAGCCCTTGAGGGAATCTCGCCAGAATTGGGC CCGACGCTGGACACGTTGCAGCTCGACGTGGCGGATTTCGCAA CAACCATCTGGCAGCAGATGGAGGAACTGGGGATGGCACCCG CGCTGCAGCCCACGCAGGGGGCAATGCCGGCCTTTGCGTCCGC GTTTCAGCGCAGGGCGGGTGGAGTCCTCGTAGCGAGCCACCTT CAATCATTTTTGGAAGTCTCGTACCGGGTGCTGAGACATCTTG CGCAGCCGTGA

	AGCGCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTT CTCTCCCTTGCACCTGTACCTCTTGGTCTTTGAATAAAGCCTGA GTAGGAAGGCGGCCGCTCGAGCATGCATCTAGA
30	mRNA sequence (transcribed) GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAAGA GCCACC AUGGCCGGUCCCGCGACCCAAAGCCCCAUGAAACUUAUGGCC CUGCAGUUGCUGCUUUGGCACUCGGCCCUCUGGACAGUCCAA GAAGCGACUCCUCUCGGACCUGCCUCAUCGUUGCCGCAGUCA UUCCUUUUGAAGUGUCUGGAGCAGGUGCGAAAGAUUCAGGG CGAUGGAGCCGCACUCCAAGAGAAGCUCUGCGCGACAUACAA ACUUUGCCAUCCCGAGGAGCUCGUACUGCUCGGGCACAGCUU GGGGAUUCCCUGGGCUCCUCUCUCGUCCUGUCCGUCGCAGGC UUUGCAGUUGGCAGGGUGCCUUUCCCAGCUCCACUCCGGUUU GUUCUUGUAUCAGGGACUGCUGCAAGCCCUUGAGGGAAUCU CGCCAGAAUUGGGCCCGACGCUGGACACGUUGCAGCUCGACG UGGCGGAUUUCGCAACAACCAUCUGGCAGCAGAUGGAGGAA CUGGGGAUGGCACCCGCGCUGCAGCCCACGCAGGGGGCAAUG CCGGCCUUUGCGUCCGCGUUUCAGCGCAGGGCGGGUGGAGUC CUCGUAGCGAGCCACCUUCAAUCAUUUUUGGAAGUCUCGUA CCGGGUGCUGAGACAUCUUGCGCAGCCGUGA AGCGCUGCCUUCUGCGGGGCUUGCCUUCUGGCCAUGCCCUUC UUCUCUCCCUUGCACCUGUACCUCUUGGUCUUUGAAUAAAGC CUGAGUAGGAAG

Example 2: PCR for cDNA Production

[00640] PCR procedures for the preparation of cDNA are performed using 2x KAPA HIFI™ HotStart ReadyMix by Kapa Biosystems (Woburn, MA). This system includes 2x KAPA ReadyMix12.5 µl; Forward Primer (10 uM) 0.75 µl; Reverse Primer (10 uM) 0.75 µl; Template cDNA 100 ng; and dH_20 diluted to 25.0 µl. The reaction conditions are at 95° C for 5 min. and 25 cycles of 98° C for 20 sec, then 58° C for 15 sec, then 72° C for 45 sec, then 72° C for 5 min. then 4° C to termination.

[00641] The reverse primer of the instant invention incorporates a poly-T_{120} for a poly-A_{120} in the mRNA. Other reverse primers with longer or shorter poly(T) tracts can be used to adjust the length of the poly(A) tail in the mRNA.

[00642] The reaction is cleaned up using Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer's instructions (up to 5 µg). Larger reactions will require a cleanup using a product with a larger capacity. Following the cleanup, the

cDNA is quantified using the NanoDrop and analyzed by agarose gel electrophoresis to confirm the cDNA is the expected size. The cDNA is then submitted for sequencing analysis before proceeding to the *in vitro* transcription reaction.

Example 3. *In vitro* Transcription (IVT)

[00643] The *in vitro* transcription reaction generates mRNA containing modified nucleotides or modified RNA. The input nucleotide triphosphate (NTP) mix is made in-house using natural and un-natural NTPs.

[00644] A typical *in vitro* transcription reaction includes the following:

Template cDNA	1.0 μg
10x transcription buffer (400 mM Tris-HCl pH 8.0, 190 mM $MgCl_2$, 50 mM DTT, 10 mM Spermidine)	2.0 μl
Custom NTPs (25mM each)	7.2 μl
RNase Inhibitor	20 U
T7 RNA polymerase	3000 U
dH_20	Up to 20.0 μl
Incubation at 37° C for 3 hr-5 hrs.	

[00645] The crude IVT mix may be stored at 4° C overnight for cleanup the next day. 1 U of RNase-free DNase is then used to digest the original template. After 15 minutes of incubation at 37° C, the mRNA is purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. This kit can purify up to 500 μg of RNA. Following the cleanup, the RNA is quantified using the NanoDrop and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred.

Example 4. Enzymatic Capping of mRNA

[00646] Capping of the mRNA is performed as follows where the mixture includes: IVT RNA 60 μg-180μg and dH_20 up to 72 μl. The mixture is incubated at 65° C for 5 minutes to denature RNA, and then is transferred immediately to ice.

[00647] The protocol then involves the mixing of 10x Capping Buffer (0.5 M Tris-HCl (pH 8.0), 60 mM KCl, 12.5 mM $MgCl_2$) (10.0 μl); 20 mM GTP (5.0 μl); 20 mM

S-Adenosyl Methionine (2.5 μl); RNase Inhibitor (100 U); 2′-O-Methyltransferase (400U); Vaccinia capping enzyme (Guanylyl transferase) (40 U); dH_20 (Up to 28 μl); and incubation at 37° C for 30 minutes for 60 μg RNA or up to 2 hours for 180 μg of RNA.

[00648] The mRNA is then purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. Following the cleanup, the RNA is quantified using the NANODROP™ (ThermoFisher, Waltham, MA) and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred. The RNA product may also be sequenced by running a reverse-transcription-PCR to generate the cDNA for sequencing.

Example 5. PolyA Tailing Reaction

[00649] Without a poly-T in the cDNA, a poly-A tailing reaction must be performed before cleaning the final product. This is done by mixing Capped IVT RNA (100 μl); RNase Inhibitor (20 U); 10x Tailing Buffer (0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl, 100 mM $MgCl_2$)(12.0 μl); 20 mM ATP (6.0 μl); Poly-A Polymerase (20 U); dH_20 up to 123.5 μl and incubation at 37° C for 30 min. If the poly-A tail is already in the transcript, then the tailing reaction may be skipped and proceed directly to cleanup with Ambion's MEGACLEAR™ kit (Austin, TX) (up to 500 μg). Poly-A Polymerase is preferably a recombinant enzyme expressed in yeast.

[00650] For studies performed and described herein, the poly-A tail is encoded in the IVT template to comprise160 nucleotides in length. However, it should be understood that the processivity or integrity of the polyA tailing reaction may not always result in exactly 160 nucleotides. Hence polyA tails of approximately 160 nucleotides, e.g, about 150-165, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164 or 165 are within the scope of the invention.

Example 6. Natural 5′ Caps and 5′ Cap Analogues

[00651] 5′-capping of modified RNA may be completed concomitantly during the *in vitro*-transcription reaction using the following chemical RNA cap analogs to generate the 5′-guanosine cap structure according to manufacturer protocols: 3′-O-Me-m7G(5')ppp(5') G [the ARCA cap];G(5')ppp(5')A; G(5')ppp(5')G; m7G(5')ppp(5')A; m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). 5′-

capping of modified RNA may be completed post-transcriptionally using a Vaccinia Virus Capping Enzyme to generate the "Cap 0" structure: m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). Cap 1 structure may be generated using both Vaccinia Virus Capping Enzyme and a 2′-O methyl-transferase to generate: m7G(5')ppp(5')G-2′-O-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2′-O-methylation of the 5′-antepenultimate nucleotide using a 2′-O methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2′-O-methylation of the 5′-preantepenultimate nucleotide using a 2′-O methyl-transferase. Enzymes are preferably derived from a recombinant source.

[00652] When transfected into mammalian cells, the modified mRNAs have a stability of between 12-18 hours or more than 18 hours, e.g., 24, 36, 48, 60, 72 or greater than 72 hours.

Example 7. Capping

A. Protein Expression Assay

[00653] Synthetic mRNAs encoding human G-CSF (mRNA sequence shown in SEQ ID NO: 30 with a polyA tail approximately 160 nucletodies in length not shown in sequence) containing the ARCA (3′ O-Me-m7G(5')ppp(5')G) cap analog or the Cap1 structure can be transfected into human primary keratinocytes at equal concentrations. 6, 12, 24 and 36 hours post-transfection the amount of G-CSF secreted into the culture medium can be assayed by ELISA. Synthetic mRNAs that secrete higher levels of G-CSF into the medium would correspond to a synthetic mRNA with a higher translationally-competent Cap structure.

B. Purity Analysis Synthesis

[00654] Synthetic mRNAs encoding human G-CSF (mRNA sequence shown in SEQ ID NO: 30 with a polyA tail approximately 160 nucletodies in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure crude synthesis products can be compared for purity using denaturing Agarose-Urea gel electrophoresis or HPLC analysis. Synthetic mRNAs with a single, consolidated band by electrophoresis correspond to the higher purity product compared to a synthetic mRNA with multiple bands or streaking bands. Synthetic mRNAs with a

single HPLC peak would also correspond to a higher purity product. The capping reaction with a higher efficiency would provide a more pure mRNA population.

C. Cytokine Analysis

[00655] Synthetic mRNAs encoding human G-CSF (mRNA sequence shown in SEQ ID NO: 30; with a polyA tail approximately 160 nucletodies in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure can be transfected into human primary keratinocytes at multiple concentrations. 6, 12, 24 and 36 hours post-transfection the amount of pro-inflammatory cytokines such as TNF-alpha and IFN-beta secreted into the culture medium can be assayed by ELISA. Synthetic mRNAs that secrete higher levels of pro-inflammatory cytokines into the medium would correspond to a synthetic mRNA containing an immune-activating cap structure.

D. Capping Reaction Efficiency

[00656] Synthetic mRNAs encoding human G-CSF (mRNA shown in SEQ ID NO: 30 with a polyA tail approximately 160 nucletodies in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure can be analyzed for capping reaction efficiency by LC-MS after capped mRNA nuclease treatment. Nuclease treatment of capped mRNAs would yield a mixture of free nucleotides and the capped 5′-5-triphosphate cap structure detectable by LC-MS. The amount of capped product on the LC-MS spectra can be expressed as a percent of total mRNA from the reaction and would correspond to capping reaction efficiency. The cap structure with higher capping reaction efficiency would have a higher amount of capped product by LC-MS.

Example 8. Agarose Gel Electrophoresis of Modified RNA or RT PCR Products

[00657] Individual modified RNAs (200-400 ng in a 20 µl volume) or reverse transcribed PCR products (200-400 ng) are loaded into a well on a non-denaturing 1.2% Agarose E-Gel (Invitrogen, Carlsbad, CA) and run for 12-15 minutes according to the manufacturer protocol.

Example 9. Nanodrop Modified RNA Quantification and UV Spectral Data

[00658] Modified RNAs in TE buffer (1 μl) are used for Nanodrop UV absorbance readings to quantitate the yield of each modified RNA from an *in vitro* transcription reaction.

Example 10. Method of Screening for Protein Expression

A. Electrospray Ionization

[00659] A biological sample which may contain proteins encoded by modified RNA administered to the subject is prepared and analyzed according to the manufacturer protocol for electrospray ionization (ESI) using 1, 2, 3 or 4 mass analyzers. A biologic sample may also be analyzed using a tandem ESI mass spectrometry system.

[00660] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

B. Matrix-Assisted Laser Desorption/Ionization

[00661] A biological sample which may contain proteins encoded by modified RNA administered to the subject is prepared and analyzed according to the manufacturer protocol for matrix-assisted laser desorption/ionization (MALDI).

[00662] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

C. Liquid Chromatography-Mass spectrometry-Mass spectrometry

[00663] A biological sample, which may contain proteins encoded by modified RNA, may be treated with a trypsin enzyme to digest the proteins contained within. The resulting peptides are analyzed by liquid chromatography-mass spectrometry-mass spectrometry (LC/MS/MS). The peptides are fragmented in the mass spectrometer to yield diagnostic patterns that can be matched to protein sequence databases via computer algorithms. The digested sample may be diluted to achieve 1 ng or less starting material for a given protein. Biological samples containing a simple buffer background (e.g. water or volatile salts) are amenable to direct in-solution digest; more complex backgrounds (e.g. detergent, non-volatile salts, glycerol) require an additional clean-up step to facilitate the sample analysis.

[00664] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

Example 11. LDL-R1 mutant mRNA sequences

[00665] Sequences encoding one or more LDL-R proteins which are deficient in PCSK9 binding are given in Table 4. The start site of the RNA is underlined "AUG" and the 5' UTR as well as the 3'UTR are bolded.

Table 4. LDL-R Sequences

Description	Sequence	SEQ ID NO
LDLR1_D331E mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUAACG AGUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGAACUCAAAAUUGGGUAUGAGUGUCUCUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG	7

	ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU** **UCUGGCCAUGCCCUUCUUCUCUCCCUUGCACCUGUA** **CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	
LDLR1_L339D mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU** **AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG	8

	GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUAACG AGUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGAUCUCAAAAUUGGGUAUGAGUGUGAUUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU** **UCUGGCCAUGCCCUUCUUCUCUCCCUUGCACCUGUA** **CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	

LDLR1_N316A mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUGCAG AGUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGAUCUCAAAAUUGGGUAUGAGUGUCUCUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG	9

	AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU UCUGGCCAUGCCCUUCUUCUCCCCUUGCACCUGUA CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	
LDLR1_E317A mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUAACG CAUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGAUCUCAAAAUUGGGUAUGAGUGUCUCUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU	10

	CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU** **UCUGGCCAUGCCCUUCUUCUCUCCCUUGCACCUGUA** **CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	
LDLR1_Y336A mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU** **AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA	11

	AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUAACG AGUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGAUCUCAAAAUUGGGGCAGAGUGUCUCUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU	

	ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU** **UCUGGCCAUGCCCUUCUUCUCUCCCUUGCACCUGUA** **CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	
LDLR1_4A mRNA	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU** **AUAAGAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUU AGAUGGACAGUCGCGCUCCUCCUUGCAGCAGCAGGAA CUGCGGUCGGAGAUCGAUGCGAGCGCAACGAGUUCCA AUGCCAAGAUGGGAAGUGUAUUUCGUACAAGUGGGUC UGCGAUGGAUCAGCGGAAUGUCAGGACGGAAGCGAUG AGAGCCAAGAAACAUGCCUCUCAGUGACAUGCAAGUC GGGAGACUUCUCGUGCGGAGGACGCGUAAACAGAUGU AUUCCACAGUUUUGGCGCUGCGAUGGUCAGGUGGACU GCGACAACGGUUCAGAUGAACAGGGAUGUCCUCCGAA AACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUGGA AAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUC GGGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCC GGUACUUACUUGCGGGCCAGCCUCAUUCCAAUGCAAC AGCUCAACGUGCAUUCCCCAGCUGUGGGCCUGUGACA AUGAUCCUGAUUGUGAGGACGGUAGCGACGAGUGGCC GCAGAGAUGUAGGGGUUUGUACGUAUUCCAAGGAGAC UCAAGCCCCUGUUCCGCCUUUGAGUUUCACUGCCUGU CGGGUGAAUGCAUCCACUCCAGCUGGCGAUGUGAUGG UGGGCCCGACUGCAAAGAUAAGAGCGACGAGGAGAAU UGCGCGGUCGCGACGUGCAGACCCGAUGAGUUCCAGU GCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAGUG UGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAG GUCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGA ACAAGUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCU CGAUAAAGUCUGCAACAUGGCGCGAGAUUGUAGGGAU UGGUCAGACGAACCCAUCAAGGAGUGCGGCACUGCAG CAUGUUUGGACAAUAACGGCGGGUGUUCGCACGUCUG CAAUGCACUCAAAAUUGGGGCAGAGUGUCUCUGUCCU GACGGAUUCCAGCUGGUCGCGCAGCGCAGAUGCGAGG ACAUCGACGAGUGCCAGGACCCCGACACAUGUUCGCA GUUGUGUGUCAACCUUGAAGGAGGGUACAAGUGCCAG UGCGAGGAGGGAUUUCAGCUUGACCCGCACACGAAAG CAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUUUUU CACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGA AUGUCGUGGCCCUUGACACGGAGGUAGCAUCAAAUAG AAUCUACUGGUCCGACCUCUCACAGAGAAUGAUCUGU UCAACACAGUUGGAUCGGGCGCACGGGGUGUCGUCGU ACGAUACGGUAAUUAGCCGCGACAUCCAGGCGCCAGA CGGACUCGCGGUCGACUGGAUCCAUAGCAACAUCUAC UGGACAGACUCCGUGUUGGGAACCGUAUCCGUAGCUG ACACAAAGGGAGUGAAGCGGAAAACUCUUUUUAGAGA GAACGGCAGCAAACCGAGAGCAAUCGUGGUCGAUCCG GUGCAUGGAUUCAUGUAUUGGACCGAUUGGGGAACGC CAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUCGA CAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCA AACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGU AUUGGGUGGACUCAAAGCUCCACUCGAUCAGCUCGAU CGACGUGAACGGCGGAAAUAGGAAAACUAUUCUCGAA	12

	GAUGAGAAAAGACUGGCCCACCCCUUCUCGCUCGCGG UGUUCGAGGACAAAGUAUUUUGGACAGACAUCAUCAA CGAAGCGAUCUUUUCAGCCAACCGCCUGACAGGGUCG GAUGUCAAUCUCUUGGCCGAAAACCUUCUGAGCCCGG AAGAUAUGGUCUUGUUUCACAAUUUGACCCAACCCAG AGGUGUGAAUUGGUGCGAACGGACGACAUUGUCGAAC GGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCCAGA UUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCC AGACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGU CUCACCGAAGCGGAAGCGGCAGUGGCCACACAAGAGA CUUCGACUGUCCGCCUUAAAGUGUCCUCGACGGCGGU CCGAACUCAGCAUACGACCACACGACCCGUGCCCGAU ACCUCGCGGUUGCCCGGAGCAACACCGGGGUUGACGA CAGUAGAAAUCGUAACCAUGAGCCACCAGGCACUUGG AGAUGUCGCAGGCAGAGGCAAUGAGAAGAAACCCAGC UCGGUCAGAGCCCUCAGCAUCGUGCUGCCUAUUGUGC UGCUUGUGUUUCUCUGUUUGGGUGUGUUCUUGUUGU GGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUUAA CUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAU GAAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGU ACCCGUCCAGGCAAAUGGUAUCACUUGAGGACGACGU GGCC**UGAUAAUAGGCUGCCUUCUGCGGGGCUUGCCU UCUGGCCAUGCCCUUCUUCUCUCCCUUGCACCUGUA CCUCUUGGUCUUUGAAUAAAGCCUGAGUAGGAAG**	
Common LDLR1 5'UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATAT AAGAGCCACC	31
Common LDLR1 3'UTR (mouse origin)	TGATAATAGGCTGCCTTCTGCGGGGCTTGCCTTCTGGCC ATGCCCTTCTTCTCTCCCTTGCACCTGTACCTCTTGGTCT TTGAATAAAGCCTGAGTAGGAAG	32

Example 12. LDLR1 protein sequences

[00666] Sequences of the one or more mutan LDL-R1 proteins are given in Table 5.

Table 5. Protein sequences

Description	**Sequence**	**SEQ ID NO**
LDLR1_D331E Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTNECLDNN GGCSHVCNELKIGYECLCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF	33

	SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	
LDLR1_L339D Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTNECLDNN GGCSHVCNDLKIGYECDCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	34
LDLR1_N316A Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTAECLDNN GGCSHVCNDLKIGYECLCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	35

LDLR1_E317A Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTNACLDNN GGCSHVCNDLKIGYECLCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	36
LDLR1_Y336A Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTNECLDNN GGCSHVCNDLKIGAECLCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	37
LDLR1_4A Protein	MGPWGWKLRWTVALLLAAAGTAVGDRCERNEFQCQDGK CISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV NRCIPQFWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDG KCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCI PQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFE FHCLSGECIHSSWRCDGGPDCKDKSDEENCAVATCRPDEFQ CSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKF KCHSGECITLDKVCNMARDCRDWSDEPIKECGTAACLDNN	38

	GGCSHVCNALKIGAECLCPDGFQLVAQRRCEDIDECQDPDT CSQLCVNLEGGYKCQCEEGFQLDPHTKACKAVGSIAYLFFT NRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSD LSQRMICSTQLDRAHGVSSYDTVISRDIQAPDGLAVDWIHS NIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPV HGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGIT LDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDEKRLAHPF SLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPED MVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSP KFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS STAVRTQHTTTRPVPDTSRLPGATPGLTTVEIVTMSHQALG DVAGRGNEKKPSSVRALSIVLPIVLLVFLCLGVFLLWKNWR LKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSL EDDVA	

Example 13. Cyp7a1 sequence

[00667] Sequences encoding CYP7A1 protein open reading frame, and the 5'UTR and 3'UTR are given in Table 6. Also shown is the sequence of the encoded protein.

Table 6. CYP7a1 sequences

Description	Sequence	SEQ ID NO
CYP7a1 Coding Region	ATGATGACCACATCTTTGATTTGGGGGATTGCTATAGCAGCA TGCTGTTGTCTATGGCTTATTCTTGGAATTAGGAGAAGGCAA ACGGGTGAACCACCTCTTGAGAATGGATTAATTCCATACCTG GGCTGTGCTCTGCAATTTGGTGCCAATCCTCTTGAGTTCCTC AGAGCAAATCAAAGGAAACATGGTCATGTTTTTACCTGCAA ACTAATGGGAAAATATGTCCATTTCATCACAAATCCCTTGTC ATACCATAAGGTGTTGTGCCACGGAAAATATTTTGATTGGAA AAAATTTCACTTTGCTACTTCTGCGAAGGCATTTGGGCACAG AAGCATTGACCCGATGGATGGAAATACCACTGAAAACATAA ACGACACTTTCATCAAAACCCTGCAGGGCCATGCCTTGAATT CCCTCACGGAAAGCATGATGGAAAACCTCCAACGTATCATG AGACCTCCAGTCTCCTCTAACTCAAAGACCGCTGCCTGGGTG ACAGAAGGGATGTATTCTTTCTGCTACCGAGTGATGTTTGAA GCTGGGTATTTAACTATCTTTGGCAGAGATCTTACAAGGCGG GACACACAGAAAGCACATATTCTAAACAATCTTGACAACTT CAAGCAATTCGACAAAGTCTTTCCAGCCCTGGTAGCAGGCCT CCCCATTCACATGTTCAGGACTGCGCACAATGCCCGGGAGA AACTGGCAGAGAGCTTGAGGCACGAGAACCTCCAAAAGAG GGAAAGCATCTCAGAACTGATCAGCCTGCGCATGTTTCTCAA TGACACTTTGTCCACCTTTGATGATCTGGAGAAGGCCAAGAC ACACCTCGTGGTCCTCTGGGCATCGCAAGCAAACACCATTCC AGCGACTTTCTGGAGTTTATTTCAAATGATTAGGAACCCAGA AGCAATGAAAGCAGCTACTGAAGAAGTGAAAAGAACATTA GAGAATGCTGGTCAAAAAGTCAGCTTGGAAGGCAATCCTAT TTGTTTGAGTCAAGCAGAACTGAATGACCTGCCAGTATTAGA TAGTATAATCAAGGAATCGCTGAGGCTTTCCAGTGCCTCCCT CAACATCCGGACAGCTAAGGAGGATTTCACTTTGCACCTTGA GGACGGTTCCTACAACATCCGAAAAGATGACATCATAGCTC	39

	TTTACCCACAGTTAATGCACTTAGATCCAGAAATCTACCCAG ACCCTTTGACTTTTAAATATGATAGGTATCTTGATGAAAACG GGAAGACAAAGACTACCTTCTATTGTAATGGACTCAAGTTA AAGTATTACTACATGCCCTTTGGATCGGGAGCTACAATATGT CCTGGAAGATTGTTCGCTATCCACGAAATCAAGCAATTTTTG ATTCTGATGCTTTCTTATTTTGAATTGGAGCTTATAGAGGGC CAAGCTAAATGTCCACCTTTGGACCAGTCCCGGGCAGGCTTG GGCATTTTGCCGCCATTGAATGATATTGAATTTAAATATAAA TTCAAGCATTTG	
CYP7a1 5'UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAG AGCCACC	40
CYP7a1 3'UTR	TGATAATAGGCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCT TGGGCCTCCCCCCAGCCCCTCCTCCCCTTCCTGCACCCGTAC CCCCGTGGTCTTTGAATAAAGTCTGAGTGGGCGGC	41
CYP7A1 Protein	MMTTSLIWGIAIAACCCLWLILGIRRRQTGEPPLENGLIPYLGCA LQFGANPLEFLRANQRKHGHVFTCKLMGKYVHFITNPLSYHKV LCHGKYFDWKKFHFATSAKAFGHRSIDPMDGNTTENINDTFIK TLQGHALNSLTESMMENLQRIMRPPVSSNSKTAAWVTEGMYS FCYRVMFEAGYLTIFGRDLTRRDTQKAHILNNLDNFKQFDKVF PALVAGLPIHMFRTAHNAREKLAESLRHENLQKRESISELISLR MFLNDTLSTFDDLEKAKTHLVVLWASQANTIPATFWSLFQMIR NPEAMKAATEEVKRTLENAGQKVSLEGNPICLSQAELNDLPVL DSIIKESLRLSSASLNIRTAKEDFTLHLEDGSYNIRKDDIIALYPQ LMHLDPEIYPDPLTFKYDRYLDENGKTKTTFYCNGLKLKYYY MPFGSGATICPGRLFAIHEIKQFLILMLSYFELELIEGQAKCPPLD QSRAGLGILPPLNDIEFKYKFKHL	23

Example 14. NASH HCC Animal Model

[00668] Compounds including polynucleotides, primary constructs and mmRNA of the invention may be tested in animal models for non-alcololic steatohepatitis (NASH). One model involves the use of STAM(TM) mice (Stelic Institute and Co, Tokyo Japan).

Materials for Examples 15-20

[00669] Sequences encoding LDLR protein open reading frame, mCherry protein open reading frame, and luciferase protein open reading frame and the 5'UTR and 3'UTR are given in Table 7. The start site of the RNA is underlined "AUG" and the 5' UTR as well as the 3'UTR are bolded.

Table 7. LDLR, mCherry and Luciferase Sequences

Name	Sequence	SEQ ID NO
LDLR mRNA sequence	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAA GAGCCACC**AUGGGUCCGUGGGGCUGGAAGCUUAGAUGGAC AGUCGCGCUCCUCCUUGCAGCAGCAGGAACUGCGGUCGGAG AUCGAUGCGAGCGCAACGAGUUCCAAUGCCAAGAUGGGAA	42

	GUGUAUUUCGUACAAGUGGGUCUGCGAUGGAUCAGCGGAA UGUCAGGACGGAAGCGAUGAGAGCCAAGAAACAUGCCUCUC AGUGACAUGCAAGUCGGGAGACUUCUCGUGCGGAGGACGC GUAAACAGAUGUAUUCCACAGUUUUGGCGCUGCGAUGGUC AGGUGGACUGCGACAACGGUUCAGAUGAACAGGGAUGUCC UCCGAAAACGUGCUCACAAGACGAGUUUCGCUGCCAUGAUG GAAAGUGCAUUUCGCGGCAGUUCGUAUGUGAUUCGGAUCG GGACUGUCUGGACGGCUCGGACGAAGCGUCAUGCCCGGUAC UUACUUGCGGGCCAGCCUCAUUCCAAUGCAACAGCUCAACG UGCAUUCCCCAGCUGUGGGCCUGUGACAAUGAUCCUGAUUG UGAGGACGGUAGCGACGAGUGGCCGCAGAGAUGUAGGGGU UUGUACGUAUUCCAAGGAGACUCAAGCCCCUGUUCCGCCUU UGAGUUUCACUGCCUGUCGGGUGAAUGCAUCCACUCCAGCU GGCGAUGUGAUGGUGGGCCCGACUGCAAAGAUAAGAGCGA CGAGGAGAAUUGCGCGGUCGCGACGUGCAGACCCGAUGAGU UCCAGUGCUCCGAUGGAAACUGCAUCCACGGGAGCCGGCAG UGUGAUCGCGAGUACGAUUGUAAAGACAUGUCAGACGAGG UCGGAUGCGUGAACGUCACGUUGUGCGAGGGUCCGAACAA GUUUAAGUGCCAUUCGGGCGAAUGUAUUACGCUCGAUAAA GUCUGCAACAUGGCGCGAGAUUGUAGGGAUUGGUCAGACG AACCCAUCAAGGAGUGCGGCACUAACGAGUGUUUGGACAA UAACGGCGGGUGUUCGCACGUCUGCAAUGAUCUCAAAAUU GGGUAUGAGUGUCUCUGUCCUGACGGAUUCCAGCUGGUCGC GCAGCGCAGAUGCGAGGACAUCGACGAGUGCCAGGACCCCG ACACAUGUUCGCAGUUGUGUGUCAACCUUGAAGGAGGGUA CAAGUGCCAGUGCGAGGAGGGAUUUCAGCUUGACCCGCACA CGAAAGCAUGUAAAGCGGUGGGGUCCAUUGCGUAUUUGUU UUUCACAAACAGACAUGAAGUGCGGAAGAUGACCCUUGAU CGCAGCGAAUAUACGUCACUGAUCCCUAAUCUUAGGAAUGU CGUGGCCCUUGACACGGAGGUAGCAUCAAAUAGAAUCUACU GGUCCGACCUCUCACAGAGAAUGAUCUGUUCAACACAGUUG GAUCGGGCGCACGGGGUGUCGUCGUACGAUACGGUAAUUA GCCGCGACAUCCAGGCGCCAGACGGACUCGCGGUCGACUGG AUCCAUAGCAACAUCUACUGGACAGACUCCGUGUUGGGAAC CGUAUCCGUAGCUGACACAAAGGGAGUGAAGCGGAAAACU CUUUUUAGAGAGAACGGCAGCAAACCGAGAGCAAUCGUGG UCGAUCCGGUGCAUGGAUUCAUGUAUUGGACCGAUUGGGG AACGCCAGCCAAAAUCAAGAAAGGCGGUUUGAAUGGGGUC GACAUCUACUCGCUGGUGACUGAGAAUAUUCAGUGGCCAA ACGGGAUCACCUUGGACUUGUUGUCGGGGAGGUUGUAUUG GGUGGACUCAAAGCUCCACUCGAUCAGCUCGAUCGACGUGA ACGGCGGAAAUAGGAAAACUAUUCUCGAAGAUGAGAAAAG ACUGGCCCACCCCUUCUCGCUCGCGGUGUUCGAGGACAAAG UAUUUUGGACAGACAUCAUCAACGAAGCGAUCUUUUCAGCC AACCGCCUGACAGGGUCGGAUGUCAAUCUCUUGGCCGAAAA CCUUCUGAGCCCGGAAGAUAUGGUCUUGUUUCACAAUUUG ACCCAACCCAGAGGUGUGAAUUGGUGCGAACGGACGACAUU GUCGAACGGAGGUUGCCAGUAUCUCUGUCUCCCUGCACCCC AGAUUAAUCCCCAUUCACCCAAGUUCACGUGUGCGUGCCCA GACGGAAUGCUUCUUGCGAGGGACAUGAGAUCCUGUCUCAC CGAAGCGGAAGCGGCAGUGGCCACACAAGAGACUUCGACUG UCCGCCUUAAAGUGUCCUCGACGGCGGUCCGAACUCAGCAU ACGACCACACGACCCGUGCCCGAUACCUCGCGGUUGCCCGG AGCAACACCGGGGUUGACGACAGUAGAAAUCGUAACCAUG AGCCACCAGGCACUUGGAGAUGUCGCAGGCAGAGGCAAUGA	

	GAAGAAACCCAGCUCGGUCAGAGCCCUCAGCAUCGUGCUGC CUAUUGUGCUGCUUGUGUUUCUCUGUUUGGGUGUGUUCUU GUUGUGGAAGAACUGGCGCCUUAAGAAUAUCAACUCGAUU AACUUCGAUAAUCCGGUAUACCAGAAAACCACAGAGGAUG AAGUGCAUAUUUGUCACAACCAAGAUGGCUAUUCGUACCCG UCCAGGCAAAUGGUAUCACUUGAGGACGACGUGGCCUGA**U** **AAGCUGCCUUCUGCGGGGCUUGCCUUCUGGCCAUGCCCU** **UCUUCUCUCCCUUGCACCUGUACCUCUUGGUCUUUGAAU** **AAAGCCUGAGUAGGAAG**	
mCherry mRNA sequence	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAA** **GAGCCACC**AUGGUAUCCAAGGGGGAGGAGGACAACAUGGC GAUCAUCAAGGAGUUCAUGCGAUUCAAGGUGCACAUGGAA GGUUCGGUCAACGGACACGAAUUUGAAAUCGAAGGAGAGG GUGAAGGAAGGCCCUAUGAAGGGACACAGACCGCGAAACUC AAGGUCACGAAAGGGGGACCACUUCCUUUCGCCUGGGACAU UCUUUCGCCCCAGUUUAUGUACGGGUCCAAAGCAUAUGUGA AGCAUCCCGCCGAUAUUCCUGACUAUCUGAAACUCAGCUUU CCCGAGGGAUUCAAGUGGGAGCGGGUCAUGAACUUUGAGG ACGGGGGUGUAGUCACCGUAACCCAAGACUCAAGCCUCCAA GACGGCGAGUUCAUCUACAAGGUCAAACUGCGGGGGACUA ACUUUCCGUCGGAUGGGCCGGUGAUGCAGAAGAAAACGAU GGGAUGGGAAGCGUCAUCGGAGAGGAUGUACCCAGAAGAU GGUGCAUUGAAGGGGGAGAUCAAGCAGAGACUGAAGUUGA AAGAUGGGGGACAUUAUGAUGCCGAGGUGAAAACGACAUA CAAAGCGAAAAAGCCGGUGCAGCUUCCCGGAGCGUAUAAUG UGAAUAUCAAGUUGGAUAUUACUUCACACAAUGAGGACUA CACAAUUGUCGAACAGUACGAACGCGCUGAGGGUAGACACU CGACGGGAGGCAUGGACGAGUUGUACAAAUGA**UAAGCUGC** **CUUCUGCGGGGCUUGCCUUCUGGCCAUGCCCUUCUUCUC** **UCCCUUGCACCUGUACCUCUUGGUCUUUGAAUAAAGCCU** **GAGUAGGAAG**	43
Luciferase mRNA sequence	**GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAA** **GAGCCACC**AUGGAAGAUGCGAAGAACAUCAAGAAGGGACC UGCCCCGUUUUACCCUUUGGAGGACGGUACAGCAGGAGAAC AGCUCCACAAGGCGAUGAAACGCUACGCCCUGGUCCCCGGA ACGAUUGCGUUUACCGAUGCACAUAUUGAGGUAGACAUCA CAUACGCAGAAUACUUCGAAAUGUCGGUGAGGCUGGCGGA AGCGAUGAAGAGAUAUGGUCUUAACACUAAUCACCGCAUC GUGGUGUGUUCGGAGAACUCAUUGCAGUUUUUCAUGCCGG UCCUUGGAGCACUUUUCAUCGGGGUCGCAGUCGCGCCAGCG AACGACAUCUACAAUGAGCGGGAACUCUUGAAUAGCAUGG GAAUCUCCCAGCCGACGGUCGUGUUUGUCUCCAAAAAGGGG CUGCAGAAAAUCCUCAACGUGCAGAAGAAGCUCCCCAUUAU UCAAAAGAUCAUCAUUAUGGAUAGCAAGACAGAUUACCAA GGGUUCCAGUCGAUGUAUACCUUUGUGACAUCGCAUUUGCC GCCAGGGUUUAACGAGUAUGACUUCGUCCCCGAGUCAUUUG ACAGAGAUAAAACCAUCGCGCUGAUUAUGAAUUCCUCGGG UAGCACCGGUUUGCCAAAGGGGGUGGCGUUGCCCCACCGCA CUGCUUGUGUGCGGUUCUCGCACGCUAGGGAUCCUAUCUUU GGUAAUCAGAUCAUUCCCGACACAGCAAUCCUGUCCGUGGU ACCUUUUCAUCACGGUUUUGGCAUGUUCACGACUCUCGGCU AUUUGAUUUGCGGUUUCAGGGUCGUACUUAUGUAUCGGUU CGAGGAAGAACUGUUUUUGAGAUCCUUGCAAGAUUACAAG AUCCAGUCGGCCCUCCUUGUGCCAACGCUUUUCUCAUUCUU UGCGAAAUCGACACUUAUUGAUAAGUAUGACCUUUCCAAU	44

	CUGCAUGAGAUUGCCUCAGGGGGAGCGCCGCUUAGCAAGGA AGUCGGGGAGGCAGUGGCCAAGCGCUUCCACCUUCCCGGAA UUCGGCAGGGAUACGGGCUCACGGAGACAACAUCCGCGAUC CUUAUCACGCCCGAGGGUGACGAUAAGCCGGGAGCCGUCGG AAAAGUGGUCCCCUUCUUUGAAGCCAAGGUCGUAGACCUCG ACACGGGAAAAACCCUCGGAGUGAACCAGAGGGGCGAGCUC UGCGUGAGAGGGCCGAUGAUCAUGUCAGGUUACGUGAAUA ACCCUGAAGCGACGAAUGCGCUGAUCGACAAGGAUGGGUG GUUGCAUUCGGGAGACAUUGCCUAUUGGGAUGAGGAUGAG CACUUCUUUAUCGUAGAUCGACUUAAGAGCUUGAUCAAAU ACAAAGGCUAUCAGGUAGCGCCUGCCGAGCUCGAGUCAAUC CUGCUCCAGCACCCCAACAUUUUCGACGCCGGAGUGGCCGG GUUGCCCGAUGACGACGCGGGUGAGCUGCCAGCGGCCGUGG UAGUCCUCGAACAUGGGAAAACAAUGACCGAAAAGGAGAU CGUGGACUACGUAGCAUCACAAGUGACGACUGCGAAGAAAC UGAGGGGAGGGGUAGUCUUUGUGGACGAGGUCCCGAAAGG CUUGACUGGGAAGCUUGACGCUCGCAAAAUCCGGGAAAUCC UGAUUAAGGCAAAGAAAGGCGGGAAAAUCGCUGUCUGA**UA** **AGCUGCCUUCUGCGGGGCUUGCCUUCUGGCCAUGCCCUU** **CUUCUCUCCCUUGCACCUGUACCUCUUGGUCUUUGAAUA** **AAGCCUGAGUAGGAAG**	

Example 15. LDLR *In Vivo* Study in Mammals

[00670] Low density lipoprotein (LDL) receptor (LDLR) mRNA (mRNA sequence shown in SEQ ID NO: 42; fully modified with 5-methylcytosine and pseudouridine; 5'cap, Cap1; polyA tail of 160 nucleotides not shown in the sequence) was complexed with Lipofectamine 2000 by mixing 8.0 μg mRNA with Dulbecco's modified Eagle's medium (DMEM) to a final volume of 0.2 mL.

[00671] Lipofectamine 2000 was diluted 12.5-fold with DMEM and mixed with an equivalent volume of the diluted LDL receptor mRNA solution. The samples were incubated 5 minutes at room temperature and a 0.1 mL volume of the complexed mRNA mixture was injected into the tail vein of each of three C57BL/6 mice. Each animal received a total dose of 2.0 μg of LDL receptor mRNA. After 6 hours, the animals were sacrificed and the spleens were removed. Splenocytes were isolated according to standard procedures (with no prior lysis of red blood cells) and stained with equivalent amounts of either IgG specific for the human LDL receptor or non-immune IgG as a control.

[00672] The expression of the LDL receptors was assessed by flow cytometry with gating on the CD11b+ splenocyte population. As shown in Figure 4, the expression of LDL receptors in the CD11b+ splenocyte population *in vivo* was evident in each of

three separate mice by the presence of rightward shifted peaks that were stained with LDL receptor IgG (LDLR IgG) as compared to cells stained with non-immune IgG (non-immune IgG).

[00673] For mice treated with Lipofectamine alone, no LDL receptor specific peak was observed and staining was similar to that observed with non-immune IgG.

Example 16. *In vivo* expression of LDLR in mice

[00674] LDLR -/- mice are used to test the in vivo expression of LDLR mmRNA. LDLR mmRNA is administered to LDLR -/- mice by injection. Tissues from the mice are examined for LDLR expression. Western blot analysis of mouse tissues is carried out to look for LDLR protein expression as a result of LDLR mmRNA administration. Real time RT-PCR is carried out on mouse tissues to look for LDLR gene expression.

Example 17. Confirmation of Peptide Identity

[00675] Proteins can be evaluated using liquid chromatography-mass spectrometry in tandem with mass spectrometry (LC-MS/MS) with quantitative LC-multiple reaction monitoring (MRM) in order to confirm the identity of the peptide.

[00676] The identity of any protein target described herein can be evaluated using the liquid chromatography-mass spectrometry in tandem with mass spectrometry (LC-MS/MS) with quantitative LC-multiple reaction monitoring (MRM) Assay (Biognosys AG, Schlieren Switzerland). HeLa cell lysates containing protein expressed from modified mRNA are evaluated using LC-MS/MS with quantitative LC-MRM Assay (Biognosys, Schlieren Switzerland) in order to confirm the identity of the peptides in the cell lysates. The identified peptide fragments are compared against known proteins including isoforms using methods known and/or described in the art.

A. Sample Preparation

[00677] Protein in each sample in lysis buffer is reduced by incubation for 1 hour at 37°C with 5 mM tris(2-carboxyethyl)phosphine (TCEP). Alkylation is carried out using 10 mM iodoacetamide for 30 minutes in the dark at room temperature. Proteins are digested to peptides using trypsin (sequence grade, PromegaCorporation, Madison, WI) at a protease: protein ratio of 1:50. Digestion is carried out overnight

at 37°C (total digestion time is 12 hours). Peptides are cleaned up for mass spectrometric analysis using C18 spin columns (The Nest Group, Southborough, MA) according to the manufacturer's instructions. Peptides are dried down to complete dryness and resuspended in LC solvent A (1% acetonitrile, 0.1% formic acid (FA)). All solvents are HPLC-grade from SIGMA-ALDRICH® (St. Louis, MO) and all chemicals, where not stated otherwise, are obtained from SIGMA-ALDRICH® (St. Louis, MO).

B. LC-MS/MS and LC-MRM

[00678] Peptides are injected to a packed C18 column (Magic AQ, 3 um particle size, 200 Å pore size, Michrom Bioresources, Inc (Auburn, CA); 11 cm column length, 75 um inner diameter, New Objective (Woburn, MA)) on a Proxeon Easy nLC nano-liquid chromatography system for all mass spectrometric analysis. LC solvents are A: 1 % acetonitrile in water with 0.1% FA; B: 3% water in acetonitrile with 0.1% FA. The LC gradient for shotgun analysis is 5-35% solvent B in 120 minutes followed by 35-100 % solvent B in 2 minutes and 100 % solvent B for 8 minutes (total gradient length is 130 minutes). LC-MS/MS shotgun runs for peptide discovery are carried out on a Thermo Scientific (Thermo Fisher Scientific) (Billerica, MA) Q Exactive mass spectrometer equipped with a standard nano-electrospray source. The LC gradient for LC-MRM is 5-35% solvent B in 30 minutes followed by 35-100% solvent B in 2 minutes and 100% solvent B for 8 minutes (total gradient length is 40 minutes). The Thermo Scientific (Thermo Fisher Scientific) (Billerica, MA) TSQ Vantage triple quadrupole mass spectrometer is equipped with a standard nano-electrospray source. In unscheduled MRM mode for recalibration it is operated at a dwell time of 20 ms per transition. For relative quantification of the peptides across samples, the TSQ Vantage is operated in scheduled MRM mode with an acquisition window length of 4 minutes. The LC eluent is electrosprayed at 1.9 kV and MRM analysis is performed using a Q1 peak width of 0.7 Da. Collision energies are calculated for the TSQ Vantage by a linear regression according to the vendor's specifications.

C. Assay Design, Data Processing and Analysis

[00679] For the generation of LC-MRM assays, the 12 most intense fragment ions from LC-MS/MS analysis are measured in scheduled LC-MRM mode and data were processed using MQUEST® (Cluetec, Karlsruhe, Germany), the scoring part of mProphet (Reiter et al, mProphet: Automated data processing and statistical validation for large-scale SRM experiments, Nature Methods, 2011 (8), 430-435; the contents of which are herein incorporated by reference). Assays were validated manually, exact fragment intensities are determined and iRTs (indexed retention times) are assigned relative to Biognosys's iRT-peptides (Escher et al. Using iRT, a normalized retention time for more targeted measurement of peptides, Proteomics, 2012 (12), 1111-1121; the contents of which are herein incorporated by reference).

[00680] For the relative quantification of the peptides across the sample series the 8 most intense transitions of each assay are measured across the sample series. Data analysis is carried out using SpectroDive™ (Biognosys, Schlieren Switzerland). Total peak areas are compared for the selected peptides and a false discover rate of 0.05 is applied. Peptides with a Qvalue below 0.05 are excluded and considered not detected in the respective sample.

Example 18. Confirmation of Peptide Identity from Modified mRNA Containing Chemical Modifications

[00681] Cell lysates containing protein produced from low density lipoprotein receptor (LDLR) modified mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and pseudouridine), were evaluated using the LC-MS/MS with quantitative LC-MRM as described in Example 17. Peptide fragments identified for the evaluated proteins are shown in Table 8. All peptides were specific for parent protein and LDLR and HFE2 was specific for the parent protein and its isoforms. In Table 8, "Uniprot ID" refers to the protein identifier from the UniProt database when the peptide fragment sequences were blasted against all review proteins in the database. Housekeeping proteins used to evaluate the protein in the cell lysates are shown in Table 9.

Table 8. Protein and Peptide Fragment Sequences

Protein	Peptide Fragment Sequence	Peptide Fragment SEQ ID NO	Uniprot ID
LDLR	MICSTQLDR	45	P01130, P01130-4, P01130-3, P01130-2
LDLR	LAHPFSLAVFEDK	46	P01130, P01130-4, P01130-3, P01130-2
LDLR	NVVALDTEVASNR	47	P01130, P01130-4, P01130-3, P01130-2
LDLR	TCSQDEFR	48	P01130, P01130-4

Table 9. Housekeeping Proteins

Protein	Peptide Fragment Sequence	Peptide Fragment SEQ ID NO	Uniprot ID
Beta-actin (ACTB)	VAPEEHPVLLTEAPLNPK	49	P60709
Glyceraldehyde-3-phosphate dehydrogenase (G3P)	VVDLMAHMASK	50	P04406
Heat shock protein HSP 90-beta (HS90B)	HLEINPDHPIVETLR	51	P08238
Heat shock protein HSP 90-beta (HS90B)	YIDQEELNK	52	P08238
L-lactate dehydrogenase A chain (LDHA)	DQLIYNLLK	53	P00338
L-lactate dehydrogenase A chain (LDHA)	GEMMDLQHGSLFLR	54	P00338
Phosphoglycerate kinase 1 (PGK1)	ALESPERPFLAILGGAK	55	P00558
Phosphoglycerate kinase 1 (PGK1)	LGDVYVNDAFGTAHR	56	P00558

60S acidic ribosomal protein P0 (RLA0)	IIQLLDDYPK	57	P05388

Example 19. Detection of Low Density Lipoprotein Receptor Expression in Cell Culture

A. *HeLa cell transfection*

[00682] HeLa cells were plated into 24-well dishes (Corning Life Sciences, Tewksbury, MA) (7.5 x 10^4 cells/well) in Eagles Minimal Essential Medium (EMEM, Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Life Technologies, Grand Island, NY) and 1X glutamax reagent (Life Technologies, Grand Island, NY) cultured overnight under standard cell culture conditions. Transfection solutions were prepared for each well to be treated by combining 250 ng of low density lipoprotein receptor (LDLR) modified mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Cap1) fully modified with 5-methylcytosine and pseudouridine, mCherry modified mRNA (mRNA sequence shown in SEQ ID NO: 43; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) or luciferase modified mRNA (mRNA sequence shown in SEQ ID NO: 44; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) with 50 µl Opti-MEM reagent (Life Technologies, Grand Island, NY) in a first tube and 1 µl of L2000 transfection reagent (Life Technologies, Grand Island, NY) in 50 µl of Opti-MEM in a second tube. After preparation, first and second tubes were incubated at room temperature for 5 minutes before combining the contents of each. Combined transfection solutions were incubated for 15 minutes at room temperature. 100 µl of transfection solution was then added to each well. Cells were cultured for an additional 16 hours before continued analysis.

B. *LDLR detection by flow cytometry*

[00683] After transfection, medium were removed from cells and 60 µl of 0.25% trypsin (Life Technologies, Grand Island, NY) was added to each well. Cells were trypsinized for 2 minutes before the addition of 240 µl/well of trypsin inhibitor (Life Technologies, Grand Island, NY). Resulting cell solutions were transferred to 96 well plates (Corning Life Sciences, Tewksbury, MA), cells were pelleted by centrifugation

(800 x gravity for 5 minutes) and supernatants were discarded. Cell pellets were washed with PBS and resuspended in Foxp3 Fixation/Permeabilization solution (eBioscience, San Diego, CA) for 45 minutes. Cells were pelleted again by centrifugation (800 x gravity for 5 minutes) and resuspended in permeabilization buffer (eBiosciences, San Diego, CA) for 10 minutes. Cells were pelleted again by centrifugation (800 x gravity for 5 minutes) and washed in permeabilization buffer. Cells were then treated with primary antibodies directed toward LDLR, followed by phycoerythrin-labeled secondary antibodies. Labeled cells were then combined with FACS buffer (PBS with 1% bovine serum albumin and 0.1% sodium azide) and transferred to cluster tubes. As shown in Figure 5, labeled cells were then analyzed by flow cytometry using a BD Accuri (BD Biosciences, San Jose, CA).

C. *LDLR detection by immunofluorescence*

[00684] Transfected cells were washed with PBS, and treated with fixation solution (PBS with 4% formaldehyde) for 20 minutes at room temperature. Cells were then washed with PBS and treated with permeabilization/blocking solution (Tris buffered saline with 5% bovine serum albumin with 0.1% Tween-20). Cells were incubated for 2 hours at room temperature with gentle agitation before washing 3 times with PBS containing 0.05% Tween-20. Cells were then treated with or without primary antibodies (goat anti-LDLR, R&D Systems, Minneapolis, MN) or normal IgG controls for 2 hours at room temperature, washed 3 times with PBS containing 0.05% Tween-20 and treated with secondary antibody solutions containing a 1:200 dilution of donkey anti-goat IgG with fluorescent label (R&D Systems, Minneapolis, MN). Cells were again washed with PBS containing 0.05% Tween-20 and examined by fluorescence microscopy imaging. Cells transiently expressing luciferase or mCherry were examined by fluorescence microscopy without fluorescent immunostaining.

Example 20. Low Density Lipoprotein Receptor (LDLR) Expression

A. *In Vitro* LDLR expression

[00685] Human embryonic kidney epithelial (HEK293) cells (LGC standards GmbH, Wesel, Germany) were seeded on 6-well plates (BD Biosciences, San Jose, USA). HEK293 were seeded at a density of about 500,000 cells per well in 3 mL cell culture medium. Lipofectomine alone or Lipofectamine containing LDLR mRNA

(mRNA shown in SEQ ID NO: 42; fully modified with 5-methylcytosine and 1-methylpseudouridine; 5'cap, cap 1, polyA tail of approximately 160 nucleotides (not shown in sequence)) or Lipofectamine containing a control of G-CSF mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence) were added directly after seeding the cells at quantities of 4000, 800, 400, 40, and 4 ng of LDLR modified mRNA per well and incubated. After eighteen hours incubation at 37°C , the cells were washed, fixed and stained. G-CSF mRNA transfected cells were treated with anti-LDLR antibody and one set of LDLR transfected cells were treated with normal goat IgG as controls. Bound primary antibodies were detected by FACS analysis following treatment with a Phycoerythrin (PE)-labeled secondary antibody.

[00686] As shown in Figure 6A, results of the FACS analyses shows 74.8% of all gated live cells were detected to express LDLR at the 800 ng dose of LDLR mRNA. At the 40 ng dose of LDLR mRNA, 11.6% of all gated live cells were detected to express LDLR. No staining was observed in LDLR mRNA treated cells stained with the control nonimmune IgG. No LDLR positive cells were detected in cells transfected with G-CSF mRNA.

B. Protein Accumulation

[00687] Human embryonic kidney epithelial (HEK293) cells (LGC standards GmbH, Wesel, Germany) were seeded on 6-well plates (BD Biosciences, San Jose, USA). HEK293 were seeded at a density of about 500,000 cells per well in 3 mL cell culture medium. Lipofectamine or Lipofectamine containing LDLR mRNA (mRNA shown in SEQ ID NO: 42; fully modified with 5-methylcytosine and 1-methylpseudouridine; 5'cap, cap 1, polyA tail of approximately 160 nucleotides (not shown in sequence)) or Lipofectamine containing a control of G-CSF mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence) were added directly after seeding the cells per well and incubated. Fifteen hours later the transfection media was replaced with complete media. Transfected cells were harvested at 0, 2, 4, 8, 24, 48, and 72 hours after media replacement. Transfected cells were treated with anti-LDLR antibody conjugated to Phycoerythrin (PE) and one set

of LDLR transfected cells were treated with normal goat IgG conjugated to PE as controls. Bound primary antibodies were detected by FACS analysis as described above.

[00688] As shown in Figure 6B, the FACS analysis shows ~65% of all gated live cells were detected to express LDLR at the 0.0-h time point (15.0-h after transfection) after washing away the transfection media. The percent positive cells declined with time at 37°C, such that by 24h post-removal of the transfection media, LDLR was not detected.

C. BODIPY®-Labeled LDLR

[00689] To evaluate whether the expressed LDLR were functional, BODIPY®-labeled LDL (Life Technologies, Woburn, MA) was used. HEK293 cells were transfected overnight with either LDLR modified mRNA (mRNA shown in SEQ ID NO: 42; fully modified with 5-methylcytosine and 1-methylpseudouridine; 5'cap, cap 1, polyA tail of approximately 16 nucleotides (not shown in sequence)) or G-CSF modified mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence), the cells were washed and incubated with increasing amounts of BODIPY-LDL. Following incubation for 1.0-h at 37°C, the cells were washed and the binding of BODIPY-LDL was assessed by FACS. Binding of BODIPY-LDL to LDLR mRNA transfected cells was high affinity (Kd ~60 ng/mL) and saturable as shown in Figure 6C. No binding was observed, in contrast, to cells transfected with G-CSF modified mRNA.

[00690] To evaluate the LDL binding specificity it was investigated whether LDL-BODIPY binding signal could be reduced by competition with unlabeled LDL. HEK293 cells were transfected overnight with LDLR and and G-CSF mRNA as a control. 0.5 ug/mL of LDL-BODIPY was added simultaneously to the transfected cells with 0.01, 0.1, 0.5, 1.0, 10, 100 or 500 ug/mL of unlabeled BODIPY. The percent of live gated transfected cells detected as positive through flow cytometery for labeled LDL are shown in Table 10. LDL-BODIPY signal was progressively reduced as more unlabeled LDL was added.

Table 10. Percent labeled LDL staining

Unlabeled LDL concentration (ug/mL)	Labeled cells detected (%)
0	100
0.01	97.7
0.1	59.0
0.5	64.1
1	76.0
10	48.4
100	3.2
500	0.9

[00691] In competition studies, binding of BODIPY-LDL could be reduced in a dose-dependent manner by unlabeled LDL (Figure 6D). These data show that binding of BODIPY-LDL to cells expressing LDLR mRNA is saturable, specific, and of high affinity.

[00692] To assess whether expression of LDLR mRNA in vivo could reduce the levels of plasma cholesterol, LDLR knock-out mice (Jackson Laboratories, Bar harbor, Maine) were treated by either a single 0.1 mL intravenous injection of 2.0 μg of LDLR mRNA in Lipofectamine 2000 or were injected with Lipofectamine 2000 alone (shown as "Negative" in Figure 6E). After 24.0-h, serum was isolated from each mouse and fractionated by fast protein liquid chromatography (FPLC) by size exclusion chromatography. As a positive control the active protein human growth hormone (Abcam Cat# ab116162) was also used (shown as "Growth Hormone" in Figure 6E). The total cholesterol content of each fraction is shown in Figure 6E. SDS-PAGE analysis showed that apo B containing lipoproteins (VLDL, IDL and LDL) were confined to fractions 3-5. The average total cholesterol of fractions 3 through 6 was 416.1 ug for the negative control, 409.0 ug for the positive control (growth hormone) and 321.3 ug for the mice administered mRNA encoding LDLR. Relative to injection of vehicle, treatment of LDLR knockout mice with a single injection of LDLR mRNA reduced the cholesterol content in the VLDL+IDL+LDL fractions by approximately 20%. These data show that expression of LDLR mRNA in LDLR knock-out mice can reduce the serum levels of cholesterol-rich lipoproteins.

Example 21. Confirmation of Peptide Identity from Modified mRNA Containing Chemical Modifications

[00693] Cell lysates containing protein produced from low density lipoprotein receptor (LDLR) modified mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Cap1) and LDLR-PCSK9-4A modified mRNA (mRNA sequence shown in SEQ ID NO: 12; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Cap1) fully modified with 5-methylcytosine and pseudouridine (5mC and pU, fully modified with 5-methylcytosine and 1-methylpseudouridine (5mC and 1mpU), fully modified with pseudouridine (pU), fully modified with 1-methylpseudouridine (1mpU) or where 25% of the uridine residues were modified with 2-thiouridine and 25% of the cytosine residues were modified with 5-methylcytosine (s2U and 5mC) were evaluated using the LC-MS/MS with quantitative LC-MRM as described in Example 17. Peptide fragments identified for the evaluated proteins are shown in Table 11.

Table 11. Proteins and Peptide Fragment Sequences

	Peptide Fragment SEQ ID NO	5mC and pU	5mC and 1mpU	s2U and 5mC	pU	1mpU
LDLR						
AVGSIAYLFFTNR	58	YES	YES	-	YES	YES
SEYTSLIPPLR	59	-	YES	-	-	YES
LDLR-PCSK9-4A						
AVGSIAYLFFTNR	58	YES	YES	-	YES	YES
IGAECLCPDGFQLVAQR	60	YES	YES	-	-	YES
TCSQDEFR	48	YES	YES	-	-	YES

Example 22. Design and synthesis of wild type LDLR and PCSK9 binding deficient LDLR modified mRNAs

A. LDLR modified mRNA

[00694] Modified mRNA encoding LDLR (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1;

fully modified with 1-methylpseudouridine and 5-methylpseudouridine) was synthesized as described previously. The modified mRNA product was analyzed with an Agilent 2100 bioanalyzer and as shown in Figure 7, a single band at the expected size of ~2.8Kb was observed.

B. PCSK9 binding deficient LDLR modified mRNA

[00695] Modified mRNAs encoding human PCSK9 binding deficient LDLRs are synthesized as described previously. The modified mRNA encodes a PCSK9 binding deficient mutant LDLR either with a single amino acid substitution such as Y336A (SEQ ID NO.37), E317A (SEQ ID NO. 36), N316A (SEQ ID NO. 35), L339D (SEQ ID NO. 34), or D331E (SEQ ID NO. 33) or a quadruple mutation variant with the amino acid substitutions: N316A, E317A, Y336A and D331A (SEQ ID NO. 38), where, for example, "N316A" means amino aicd Asparagine at position 316 is substituted for the amino acid Alanine.. The mutated LDLR mRNAs further include chemical modification described herein. Confirmation of the modified mRNA product is done by methods known in the art such as bioanalyzer and peptide digestion.

Example 23. In Vitro Expression of LDLR modified mRNA

[00696] Human Embryonic Kidney 293 (HEK293) cells were transfected with lipofectamine alone, lipofectamine containing modified mRNA encoding LDLR (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 1-methylpseudouridine and 5-methylcytosine) or a control of lipofectamine containing modified RNA encoding G-CSF (mRNA sequence shown in SEQ ID NO: 30; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 1-methylpseudouridine and 5-methylcytosine). After an 18 hour incubation at 37°C, the cells were washed, fixed and stained with either phycoerthrin (PE)-labeled anti-human LDLR antibody (R&D Systems, Minneapolis, MN; Human LDL R Affinity Purified Polyclonal AbAF2148) or PE-labeled goat non-immune IgG (R&D Systems, Minneapolis, MN; R&D Systems Purified goat IgG R&D Systems catalog number AC-108-C). Conjugation to PE was completed with the Innova

biosciences lightning-link conjugation kit (Lightning-Link R-PE Antibody Labeling Kit Novus Biologicals catalog number: 703-0010).

[00697] The expression of human LDLR was monitored by flow cytometry. Transfection with increasing amounts (4-4000 ng) of LDLR modified mRNA increased the percent of cells that stained with the PE-labeled anti-LDLR IgG but not in the cells transfected with control of G-CSF modified mRNA. The transfection of 800 ng of modified mRNA encoding LDLR is shown in Figure 8. There was no positive staining detected with PE-labeled non-immune IgG in cells transfected with LDLR modified mRNA.

[00698] LDLR expression reached a peak at 8 to 24 hours post-transfection and declined thereafter.

Example 24. Detection of LDLR

[00699] Human Embryonic Kidney 293 (HEK293) cells were transfected with lipofectamine containing modified mRNA encoding LDLR (mRNA shown in SEQ ID NO: 42, polyA of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 1-methylpseudouridine and 5-methylcytosine) or a control of lipofectamine containing modified mRNA encoding G-CSF (mRNA sequence shown in SEQ ID NO: 30; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 1-methylpseudouridine and 5-methylcytosine). After an incubation of 16 hours at 37°C, the cells were washed and replaced with complete growth media. Cells were harvested and stained for LDLR at 0, 2, 4 and 8 hours post transfection. As shown in Figure 9, LDLR was detected in 68.1% of live cells after transfection and diminished to 27.6% at 8 hours in the absence of transfection media. In Figure 9, columns 1 and 2 were transfected with mRNA encoding LDLR and column 3 was transfected with the control mRNA encoding G-CSF. Columns 1 and 3 were stained with phycoerthrin (PE)-labeled anti-human LDLR antibody (R&D Systems, Minneapolis, MN; Human LDL R Affinity Purified Polyclonal AbAF2148) and Column 2 was stained with goat IgG conjugated to PE (R&D Systems, Minneapolis, MN; R&D Systems Purified goat IgG R&D Systems catalog number AC-108-C) as a control. Conjugation to PE was completed

with the Innova biosciences lightning-link conjugation kit (Lightning-Link R-PE Antibody Labeling Kit Novus Biologicals catalog number: 703-0010).

Example 25. In Vitro Expression of PCSK9 binding deficient LDLR modified mRNAs

[00700] Modified mRNAs encoding human and mouse wild type LDLR and human PCSK9 binding deficient LDLRs are synthesized as described herein. The modified mRNAs are transfected with HEK293 cells by methods known in the art. The expression of wild type LDLR and PCSK9 binding deficient LDLR mutants are screened after transfections to determine the highest expressing modified mRNAs.

Example 26. PCSK9 down-regulation of LDLR in Hep-G2 cells

[00701] Human Hepatocellular carcinoma (Hep-G2) cells are cultured in complete media (DMEM medium with 10% lipoprotein deficient serum (Intracel Resources, Frederick, MD)) to down regulate endogenous LDLRs. The down-regulation of LDLR expression by PCSK9 will be assessed by known methods (e.g., see Lipari et al., Furin-cleaved Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Is Active and Modulates Low Density Lipoprotein Receptor and Serum Cholesterol Levels. J Biol Chem. 2012, 287(52): 43482-43491; McNutt et al. Antagonism of Secreted PCSK9 Increases Low Density Lipoprotein Receptor Expression in HepG2 Cells. J Biol Chem. 2009. 284(16): 10561-10570; each of which is herein incorporated by reference in its entirety).

[00702] One method to assess the down-regulation of LDLR expression is transfecting Hep-G2 cells with wild type LDLR modified mRNA or PCSK9 binding deficient LDLR modified mRNAs as described herein. The Hep-G2 cells are cultured in complete media to down-regulate endogenous LDLRs prior to transfection with LDLR modified mRNA. After 24 hour incubation at 37°C, the turnover of LDLR from transfection with modified mRNA encoding wild type LDLR or PCSK9 binding deficient LDLR is assessed in the presence and absence of exogenous PCSK9 (R &D Systems, Minneapolis, MN) by western blot analysis of cell lysates (PROTEIN SIMPLE™, Santa Clara, CA) and by flow cytometry (e.g., FACS sorting). The modified mRNA encoding the most PCSK9-insensitive LDLR is determined.

Example 27. Liver Cell Transducing Formulations

[00703] Lipid nanoparticles (LNPs) are formulated using methods known in the art, described herein and/or described in PCT/US2012/069610 entitled "Modified Nucleoside, Nucleotide, and Nucleic Acid Composition," herein incorporated by reference in its entirety. The LNPs used herein can comprise the ionizable lipid DLin-KC2-DMA or the cationic lipid C12-200.

[00704] Modified mRNA encoding luciferase (e.g., SEQ ID NO: 44; polyA tail of at least 140 nucleotides not shown in sequence; 5'cap, Cap1; modified with at least one chemical modification described herein) is formulated in LNPs comprising DLin-KC2-DMA or C12-200. The formulated luciferase is administered to wild type mice and LDLR deficient mice. The expression of luciferase in the liver cells of the wild type and LDLR deficient mice is measured, using methods known in the art or described herein, at predetermined intervals after administration of the modified mRNA. Twenty minutes prior to imaging, mice are injected intraperitoneally with a D-luciferin solution at 150mg/kg. Animals are anesthetized and images are acquired with an IVIS lumina II imaging system (Perkin Elmer, Waltham, MA). Bioluminescenes are measured as total flux (photons/second).

Example 28. *In vivo* expression of LDLR in mice

[00705] LDLR -/- mice are used to test the in vivo expression of modified mRNAs encoding the wild type human or murine LDLR or encoding PCSK9 binding deficient human or murine LDLRs (collectively, "LDLR modified mRNAs"). LDLR modified mRNAs formulated in lipid nanoparticles are administered to LDLR -/- mice through 0.1 ml intravenous injections containing increasing doses of between 0.005-0.5 mg/kg (e.g. 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg) of the LDLR modified mRNAs. Mice are sacrificed at various times, such as between 2 hours and 96 hours (e.g., 2hr, 2.5hr, 3hr, 3.5hr, 4hr, 5hr, 6hr, 7hr, 8hr, 9hr, 10hr, 12hr, 24hr, 48hr, 72hr and 96hr) after the injection. The livers are excised and the cell lysates and livers are prepared for analysis. The LDLR protein expression and drug level changes in mRNA transcript are measured by western blot analysis using an anti-LDLR antibody and the expression of modified LDLR mRNA in mouse tissues is analyzed by real time RT-PCR. Serum is collected at various timepoints after

injection of the modified mRNA and analyzed for cytokine panels using a mouse LUMINEX® panel. The remainder of the sera will be fractionated by FPLC for assay of VLDL+IDL+LDL cholesterol (see the method described in Garber et al. A sensitive and convenient method for lipoprotein profile analysis of individual mouse plasma samples. Journal of Lipid Research. 2000. 14: 1020-1026; herein incorporated by reference in its entirety).

[00706] In a further study, the LDLR -/- mice are administered more than once with modified mRNAs encoding the wild type human or murine LDLR or encoding PCSK9 binding deficient human or murine LDLRs. Serum is collected at various timepoints after injection of the modified mRNA and analyzed for cytokine panels using a mouse LUMINEX® panel and assayed for VLDL+IDL+LDL cholesterol. The mice are also sacrificed and the livers are excised and cell lysates are prepared for analysis.

Example 29. *In vivo* expression of LDLR in the Watanabe (WHHL) rabbit

[00707] Watanabe (WHHL) rabbits are used to test the in vivo expression of modified mRNAs encoding the wild type human LDLR or encoding PCSK9 binding deficient human LDLRs ("LDLR modified mRNAs"). LDLR modified mRNAs formulated in lipid nanoparticles are administered through injections containing 0.005-0.5 mg/kg (e.g., 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg) of the LDLR modified mRNAs to the rabbits. The WHHL rabbits are sacrificed between 2 hours and 96 hours (e.g., 2hr, 2.5hr, 3hr, 3.5hr, 4hr, 5hr, 6hr, 7hr, 8hr, 9hr, 10hr, 12hr, 24hr, 48hr, 72hr and 96hr) after the injection and the livers are excised and cell lysates are prepared for analysis. The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the expression of LDLR in rabbit tissues is analyzed by real time RT-PCR. Serum is collected at various timepoints after injection of the modified mRNA and analyzed for cytokine panels and assayed for VLDL+IDL+LDL cholesterol.

Example 30. *In vivo* expression of LDLR in the LDLR Deficient Pigs

[00708] LDLR deficient pigs (Exemplar Genetics, Sioux Center, IA) are used to test the in vivo expression of modified mRNAs encoding the wild type human LDLR or encoding PCSK9 binding deficient human LDLRs ("LDLR modified mRNAs"). LDLR modified mRNAs formulated in lipid nanoparticles are administered through injections containing 0.005-0.5 mg/kg (e.g., 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg) of the LDLR modified mRNAs to the LDLR deficient pigs. The pigs are sacrificed between 2 hours and 96 hours (e.g., 2hr, 2.5hr, 3hr, 3.5hr, 4hr, 5hr, 6hr, 7hr, 8hr, 9hr, 10hr, 12hr, 24hr, 48hr, 72hr and 96hr) after the injection and the livers are excised and cell lysates are prepared for analysis. The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the expression of LDLR in pig tissues is analyzed by real time RT-PCR. Serum is collected at various timepoints after injection of the modified mRNA and analyzed for cytokine panels and assayed for VLDL+IDL+LDL cholesterol.

Example 31. *In vivo* Expression of LDLR in LDLR Deficient Rhesus Monkeys

[00709] LDLR deficient rhesus monkeys (Southwest National Primate Research Center, San Antonio, TX) are used to test the in vivo expression of modified mRNAs encoding the wild type human LDLR or encoding PCSK9 binding deficient human LDLRs ("LDLR modified mRNAs"). LDLR modified mRNAs formulated in lipid nanoparticles are administered through injections containing 0.005-0.5 mg/kg (e.g., 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg) of the LDLR modified mRNAs to the LDLR deficient monkeys. The monkeys are sacrificed between 2 hours and 96 hours (e.g., 2hr, 2.5hr, 3hr, 3.5hr, 4hr, 5hr, 6hr, 7hr, 8hr, 9hr, 10hr, 12hr, 24hr, 48hr, 72hr and 96hr) after the injection and the livers are excised and cell lysates are prepared for analysis. The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the expression of LDLR in monkey tissues is analyzed by real time RT-PCR. Serum is collected at various timepoints after injection of the

modified mRNA and analyzed for cytokine panels and assayed for VLDL+IDL+LDL cholesterol.

Example 32. Multi-dose studies

[00710] Studies utilizing multiple doses are designed and performed using LDLR-/- mice. The mice are injected intravenously eight times (twice a week) over 28 days with 0.5 mg/kg, 0.05 mg/kg, 0.005 mg/kg or 0.0005 mg/kg of modified LDLR mRNA encoding human or mouse wild type LDLR or encoding PCSK9 binding deficient human LDLR, formulated in a lipid nanoparticle. The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the expression of LDLR in tissues is analyzed by real time RT-PCR. Sera are collected during pre-determined time intervals and analyzed for cytokines panel and assay for VLDL+IDL+LDL cholesterol as described herein.

Example 33. Total cholesterol in wild type and LDLR knock out mice

[00711] Serum from three wild type mice (C57BL/6J) and three LDLR knock out mice (B6. 129S7-ldlrtm1Her/J, Jackson Laboratories, Bar Harbor, Maine) were collected and fractionized by FPLC. Absorbance of eluted serum fractions from wild type and LDLR knock out mice were measured at 260 and 280nm. Each fraction was analyzed for total cholesterol by the Wako cholesterol E enzymatic colometric method (Wako, Richmond, VA). As shown in Figure 10A, absorbance profiles showed three distinct protein peaks in both mouse strains (Figure 10A). In The first peak, spanning fractions 3 through 6 in Figure 10B, tested highest for cholesterol in LDLR knockout mice. The second peak, spanning fractions 7 through 12 in Figure 10B, tested highest for total cholesterol in wild type mice. The third peak, spanning fractions 14 through 18 in Figure 10B, tested low or negative for cholesterol in both mouse strains.

Example 34. Expression of wild-type LDLR and PCSK9 binding-deficient variants

[00712] Human embryonic kidney epithelial (HEK293) cells (LGC standards GmbH, Wesel, Germany) are seeded on 48-well plates (BD Biosciences, San Jose, USA). HEK293 are seeded at a density of about 60,000 cells per well in 0.2 mL cell

culture medium. Formulations containing 1 uL lipofectamine and 150 ng of wild type (WT) LDLR mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine) or 150 ng of an LDLR sequence variant mRNA or a control of G-CSF mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence) are added directly after seeding the cells at quantities of 60,000 per well and incubated.

[00713] The LDLR mRNA sequence variants prepared and tested include: a four amino acid substitution variant (4A:, N316A, E317A, D331A, and Y336A) (mRNA sequence shown in SEQ ID NO: 12; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), or the following single amino acid substitution variants Y336A (mRNA sequence shown in SEQ ID NO: 11; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), E317A (mRNA sequence shown in SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), N316A (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), L339D (mRNA sequence shown in SEQ ID NO: 8; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), D331E (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine).

[00714] As controls G-CSF mRNA transfected cells were treated with anti-LDLR antibody (R&D Systems, Minneapolis, MN; Human LDL R Affinity Purified Polyclonal AbAF2148) and one set of LDLR transfected cells were treated with normal goat IgG (R&D Systems, Minneapolis, MN; Purified goat IgG R&D Systems catalog number AC-108-C). Bound primary antibodies were detected by FACS

analysis following treatment with a Phycoerythrin (PE)-labeled antibody (R&D Systems, Minneapolis, MN). Conjugation to PE was completed with the Innova biosciences lightning-link conjugation kit (Lightning-Link R-PE Antibody Labeling Kit Novus Biologicals catalog number: 703-0010).

[00715] As shown in Figure 11, the FACS analysis shows 32% of all gated live cells were detected to express LDLR at the 150 ng dose of wild type LDLR mRNA (WT in Figure 11). Similarly, for cells transfected with the LDLR mRNA variants, between 12-33% of all gated live cells were detected to express LDLR at the 150 ng dose. No staining was observed in LDLR mRNA treated cells stained with the control non-immune IgG (LDLR WT transfect Isotype stain) and no LDLR positive cells were detected in cells transfected with G-CSF mRNA (GCSF transfect LDLR stain).

Example 35. Down-modulation of LDLR by Exogenous PCSK9

[00716] Human embryonic kidney epithelial (HEK293) cells (LGC standards GmbH, Wesel, Germany) are seeded on 48-well plates (BD Biosciences, San Jose, USA). HEK293 are seeded at a density of about 60,000 cells per well in 0.2 mL cell culture medium. Formulations containing 1 uL of lipofecatmine 2000 and 150 ng of wild type (WT) LDLR mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine) or 150 ng of an LDLR sequence variant mRNA or 150 ng of a control of G-CSF mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence) are added directly after seeding the cells at quantities of 800 ng per well and incubated in the presence and in the absence of exogenous human PCSK9 at 60 μg /mL.

[00717] The LDLR mRNA sequence variants prepared and tested include: a four amino acid substitution variant (4A: N316A, E317A, D331A, and Y336A) (mRNA sequence shown in SEQ ID NO: 12; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), or the following single amino acid substitution variants Y336A(mRNA sequence shown in SEQ ID NO: 11; polyA tail of approximately 160

nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), E317A (mRNA sequence shown in SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), N316A (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), L339D (mRNA sequence shown in SEQ ID NO: 8; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), D331E (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine).

[00718] After fifteen hours at 37°C, the transfection media were removed, the cells were washed, and were treated with anti-LDLR antibody conjugated to Phycoerythrin (PE) (R&D Systems, Minneapolis, MN; Human LDL R Affinity Purified Polyclonal AbAF2148) as described above. One set of LDLR transfected cells were treated with normal goat IgG conjugated to PE (R&D Systems, Minneapolis, MN; Purified goat IgG R&D Systems catalog number AC-108-C) and another set of untransfected cells were used as controls. Cells transfected with G-CSF modified mRNA were used as an additional negative control. Conjugation to PE was completed with the Innova biosciences lightning-link conjugation kit (Lightning-Link R-PE Antibody Labeling Kit Novus Biologicals catalog number: 703-0010). Bound primary antibodies were detected by flow cytometry as described above.

[00719] As shown in Figure 12, the FACS analysis showed that cell surface LDLR expression in cells transfected with wild-type LDLR mRNA was 51.6% of gated live cells. This value decreased to 21.5% of gated live cells when exogenous PCSK9 was added to the media during cell transfection. In contrast, each of the LDLR mRNA variants with substitutions in the PCSK9 binding domain showed less sensitivity to down-modulation by exogenous PCSK9 (Table 12) with values of percent reduction ranging from -8.1% to 29% (compared to 58% reduction observed with wild-type LDLR.

Table 12. Down-Modulation of Cell Surface LDLR Expression by Exogenous PCSK9

	% of Cells LDLR Positive		Percent Reduction in LDLR Expression
LDLR mRNA	**PCSK9 Absent**	**PCSK9 Present**	**(%)**
WT	51.6	21.5	58.3
N316A, E317A, D331A, Y336A	65.1	63.3	2.8
Y336A	73.1	52.1	28.7
E317A	71.8	51.9	27.7
N316A	60.8	65.7	-8.1
L339D	69.4	60.9	12.2
D331E	69.4	60.9	0.8
G-CSF negative control	1.06	0.76	NA

Example 36. Functionality of LDLR expressed by variant LDLR mRNA

[00720] To evaluate whether the expressed LDLR were functional, BODIPY-labeled LDL was used. 60,000 HEK293 cells per well were transfected overnight with formulations containing 1 uL lipofectamine 2000 and 150 ng encoding wild type (WT) LDLR mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine) or 150 ng of an LDLR variant mRNA or 150 of a control of G-CSF mRNA (mRNA shown in SEQ ID NO: 30; fully modified with 5-methylcytosine and pseudouridine; 5'cap, cap1; polyA tail of approximately160 nucleotides not shown in sequence).

[00721] The LDLR variant mRNA sequences prepared and tested include: a four amino acid substitution variant (4A: N316A, E317A, D331A, and Y336A) (mRNA sequence shown in SEQ ID NO: 12; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), or the following single amino acid substitution variants Y336A(mRNA sequence shown in SEQ ID NO: 11; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), E317A (mRNA sequence shown in SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence;

5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), N316A (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), L339D (mRNA sequence shown in SEQ ID NO: 8; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), D331E (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine).

[00722] The cells were washed and incubated with increasing amounts (0 ug/ml, 0.1 ug/ml, 1.0 ug/ml, 10 ug/ml or 50 ug/ml) of BODIPY-LDL. Following incubation for 1 hour at 37°C, the cells were washed and the cell associated BODIPY-LDL was assessed by flow cytometry. The contour plots are shown in Figure 13A. For wild-type (WT) and LDLR PCSK9 binding variants, the binding of BODIPY-LDL to LDLR mRNA transfected cells was evident as a right-ward shift in the gated cell population that increased with BODIPY-LDL concentration. A similar rightward shift in the gated cell population was seen for cells transfected with each of the LDLR mRNA constructs. As is shown in Figure 13B, half-maximal cell association was the same for BODIPY-LDL binding to cells transfected with either wild type or the PCSK9 binding-deficient LDLR mRNAs. For each construct, BODIPY-LDL binding was of high affinity (half-maximal binding between 0.6-0.7 ng/mL) and saturable. No BODIPY-LDL binding was observed for cells transfected with G-CSF mRNA.

Example 37. Evaluate of half-life on cell surface LDLR

[00723] To assess whether exogenous PCSK9 can modulate the apparent half-life of cell surface LDL receptors in cells transfected with LDLR mRNA, HEK293 cells were plated on 24 well plates, 130,000 cells per well, transfected with LDLR mRNA and incubated for 14 hours as described above. The cell monolayers were washed and fresh media with PCSK9 (60 μg/mL) or without PCSK9 was added. After various times of incubation at 37°C, cell surface LDLR expression was monitored by

flow cytometry using anti-LDLR antibodies as described above. As is shown in Figure 14A, cell surface LDLR in cells transfected with 300 ng of wild-type LDLR mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine) decreased in a time-dependent manner with an apparent half-life of approximately 13 hours. The addition of PCSK9 to the media of cells transfected with wild-type LDLR mRNA decreased the apparent half-life of cell surface LDLR to about 4 hours. In Figure 14A, "*" represents a significant difference by statistical test.

[00724] In contrast, the addition of PCSK9 to the media of cells transfected with 300 ng of PCSK9 binding-deficient LDLR mRNA produced little or no change in the apparent half-life of cell surface LDLR (Figures 13B-13G). The PCSK9 binding-deficient LDLR mRNA sequences prepared and tested include: a four amino acid substitution variant (4A: N316A, E317A, D331A, and Y336A) (mRNA sequence shown in SEQ ID NO: 12; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), or the following single amino acid substitution variants Y336A(mRNA sequence shown in SEQ ID NO: 11; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), E317A (mRNA sequence shown in SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), N316A (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), L339D (mRNA sequence shown in SEQ ID NO: 8; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), D331E (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine).

[00725] These data in Figures 14B-14G show that cells transfected with LDLR mRNA encoding LDLR with mutations in the PCSK9 binding site fail to respond to exogenous PCSK9, suggesting that these binding variants may have a longer half-life than wild-type LDLR in vivo and be useful for treating patients with hypercholesterolemia.

Example 38. Effect of increasing PCSK9 amount on cell surface LDLR

[00726] To evaluate the down-modulation of cell surface LDLR expression by increasing amounts of PCSK9 added to the complete cell media, MEM (GlutaMAX, Life Science Catalog#41090-036) supplemented with 10% fetal bovine serum. HEK293 cells were plated at 300,000 cells per well, incubated for 6 hours, and transfected for 15 hours with 300 ng of either wild-type LDLR mRNA (mRNA sequence shown in SEQ ID NO: 42; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine) or an LDLR mRNA encoding a PCSK9 binding variant. The PCSK9 binding variant mRNA sequences prepared and tested include the single amino acid substitution variants of N316A (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine), and D331E (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and 1-methylpseudouridine). A control of mRNA encoding UGT1A1 (mRNA sequence shown in SEQ ID NO: 61; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcyostine and 1-methylpseudouridine) was also used.

[00727] After 15 hours of incubation, the cell monolayers were washed and either buffer alone or buffer containing increasing amounts of PCSK9 were added. Cells were incubated for 5 hours and cell surface LDLR expression was measured by flow cytometry as described above. As is shown in Figure 15, cell surface LDL receptors in cells transfected with wild-type LDLR mRNA were decreased in a dose-dependent manner by PCSK9. Maximal reduction in LDLR was achieved at 20 μg/mL of

exogenous PCSK9. In contrast, PCSK9 had no effect on cell surface LDLR in cells transfected with LDLR mRNA encoding the PCSK9 binding-deficient variants N316A or D331E. No cell surface LDLR was detected in HEK293 cells transfected with mRNA encoding UGT1A1. These data show that LDLR expressed from LDLR mRNAs encoding mutations in the binding site for PCSK9 are insensitive to exogenous PCSK9.

Example 39. Liver Cell Transducing Formulations of LDLR

[00728] Lipid nanoparticles (LNPs) are formulated using methods known in the art, described herein and/or described in PCT/US2012/069610 entitled "Modified Nucleoside, Nucleotide, and Nucleic Acid Composition," herein incorporated by reference in its entirety. The LNPs used herein can comprise the ionizable lipid DLin-KC2-DMA or the cationic lipid C12-200.

[00729] Modified mRNA encoding LDLR or LDLR mutants (e.g., SEQ ID NOs: 7-12; polyA tail of at least 140 nucleotides not shown in sequence; 5'cap, Cap1; modified with at least one chemical modification described herein) is formulated in LNPs comprising DLin-KC2-DMA or C12-200. The formulated LDLR or LDLR mutants is administered to wild type mice and LDLR deficient mice. The expression of LDLR in the liver cells of the wild type and LDLR deficient mice is measured, using methods known in the art or described herein, at predetermined intervals after administration of the modified mRNA.

Example 40. Delivery of LNP formulated modified mRNA

[00730] Luciferase mRNA (mRNA sequence shown in SEQ ID NO: 44; polyA tail of at least 140 nucleotides not shown in sequence; 5'cap, Cap1) is fully modified with either 5-methylcytosine and pseudouridine, fully modified with 5-methylcytosine and 1-methylpseudouridine, fully modified with pseudouridine, fully modified with 1-methylpseudouridine or 25% of the uridine residues are modified with 2-thiouridine and 25% of the cytosine residues are modified with 5-methylcytosine. The luciferase mRNA is then formulated in a lipid nanoparticle comprising the cationic lipid DLin-KC2-DMA (KC2) or C12-200. The formulated LNP in PBS or a control of PBS alone is administered intraveneously to LDLR -/- or normal mice as outlined in Table 13. The mice are imaged at 2 hours, 8 hours, 24

hours and 48 hours after injection. Ten minutes prior to imaging, mice are injected intraperitoneally with a D-luciferin solution at 150 mg/kg. Animals are then anesthetized and images are acquired with an IVIS Lumina II imaging system (Perkin Elmer).

Table 13. Dosing Regimen

Group	Mouse Strain	Cationic Lipid	Dose (mg/kg)	mRNA dose/mouse (mg)	Injection Volume (mL)
1	LDLR-/-	KC2	0.5	0.01	0.1
2	LDLR-/-	KC2	0.05	0.001	0.1
3	Normal	KC2	0.5	0.01	0.1
4	Normal	KC2	0.05	0.001	0.1
5	LDLR-/-	C12-200	0.5	0.01	0.1
6	LDLR-/-	C12-200	0.05	0.001	0.1
7	Normal	C12-200	0.5	0.01	0.1
8	Normal	C12-200	0.05	0.001	0.1
9	LDLR-/-	none	none	none	0.1

Example 41. Studies of Mammals Adminsitered UGT1A1 modified mRNA

A. *Rodents*

[00731] Studies utilizing multiple doses are designed and performed using rats and/or mice (e.g. LDLR-/- mice). The rodents are injected intramuscularly or intravenously more than once over a period of 7 days with 0.5 mg/kg, 0.05 mg/kg, 0.005 mg/kg or 0.0005 mg/kg of modified LDLR mRNA encoding human, mouse or rat LDLR or its isoforms or variants descrbed herein. The LDLR mRNA is formulated in either 5% sucrose, saline or a lipid nanoparticle. The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the drug level and transcript of LDLR in tissues is analyzed by real time RT-PCR. Sera from the rats are collected during pre-determined time intervals and analyzed for cytokines panel and analyzed for cytokines panel and assay for cholesterol levels as described herein.

B. *Non-Human Primates (NHP)*

[00732] LDLR modified mRNA formulated in 5% sucrose, saline or a lipid nanoparticle is administered to non-human primates intramuscularly or intravenously.

The injection contains a dose of LDLR mRNA of between 0.005-0.5 mg/kg (e.g. 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg). The LDLR protein expression and drug level changes in mRNA transcript are measured by western blot analysis using an anti-LDLR antibody and the level of modified LDLR mRNA in the muscle tissues are analyzed by real time RT-PCR. Sera from the non-human primates are collected at predetermined intervals after injection and analyzed for cytokines panel and assay for cholesterol levels as described herein.

Example 42. Repeat Dose Adminstration Studies of UGT1A1 Modified mRNA in Mammals

A. *Rodents*

[00733] Studies utilizing multiple doses are designed and performed using rats and/or mice (e.g. LDLR-/- mice). The rodents are injected intramuscularly or intravenously more than once (e.g., daily, twice a week, every 5 days, weekly, every 10 days, bi-weekly) over a period of 4 weeks with 0.5 mg/kg, 0.05 mg/kg, 0.005 mg/kg or 0.0005 mg/kg of modified LDLR mRNA encoding human or rat LDLR. The LDLR mRNA is formulated in either 5% sucrose, saline or a lipid nanoparticle.

[00734] The LDLR protein expression and changes in mRNA transcript is measured in the cell lysates by western blot analysis using an anti-LDLR antibody and the drug level and transcript of LDLR in tissues is analyzed by real time RT-PCR. Sera from the rats are collected during pre-determined time intervals and analyzed for cytokines panel and assay for cholesterol levels as described herein.

B. *Non-Human Primates (NHP)*

[00735] LDLR modified mRNA formulated in 5% sucrose, saline or a lipid nanoparticle is administered to non-human primates intramuscularly or intravenously more than once (e.g., daily, twice a week, every 5 days, weekly, every 10 days, bi-weekly) over a period of 4 weeks. The injection contains a dose of LDLR mRNA of between 0.005-0.5 mg/kg (e.g. 0.005mg/kg, 0.010mg/kg, 0.015mg/kg, 0.020mg/kg, 0.030 mg/kg, 0.040mg/kg, 0.050 mg/kg, 0.1mg/kg, 0.2mg/kg, 0.3mg/kg, 0.4mg/kg and 0.5mg/kg).

[00736] The non-human primates are weighed prior to the start of the study and weighed at day 8, day 15 and at the end of the study. The LDLR protein expression and drug level changes in mRNA transcript are measured by western blot analysis using an anti-LDLR antibody and the level of modified LDLR mRNA in the muscle tissues are analyzed by real time RT-PCR. Sera from the non-human primates are collected at predetermined intervals after injection and analyzed for cytokines panel and assay for cholesterol levels as described herein.

Example 43. Microphysiological Systems

[00737] The modified mRNA encoding LDLR and its variants described herein are formulated using one of the methods described herein such as in buffer, lipid nanoparticles and PLGA. These formulations are then administered to or contacted with microphysiological systems created from organ chips as described in International Publication Nos. WO2013086502, WO2013086486 and WO2013086505, the contents of each of which are herein incorporated by reference in its entirety.

Example 44. LDLR Mutations

[00738] In one embodiment, the polynucleotides described herein encode at least one LDLR protein which is deficient is binding to PCSK9. As a non-limiting example, the LDLR protein may comprise at least one mutation to be PCSK9 binding deficient as described herein.

[00739] In one embodiment, the polynucleotides described herein may be deficient in binding to disable homolog 2, mitogen-responsive phosphoprotein (DAB2). While not wishing to be bound by theory, the DAB2 binding-deficient LDLR may limit the internalization of LDLR through the DAB2 pathway and thus reducing LDLR uptake.

[00740] In one embodiment, the NPXY motif of LDLR may be modified in order to alter the signal for rapid endocytosis through coated pits of LDLR. The NPXY motif may comprise at least one mutation, at least two mutations, at least three mutations, at least four mutations or more than four mutations. As a non-limiting example, the NPXY motif may comprise amino acid 822 through amino acid 829 of a LDLR sequence. As another non-limiting example, the NPXY motif may comprise the sequence NFDNPVYQ (SEQ ID NO: 62).

[00741] In one embodiment, the LDLR sequence does not comprise a mutation in the NPXY motif. In another embodiment, the LDLR sequence may comprise a mutation but the mutation may not be at position 822, 826, 827 or 828 of LDLR where amino acid 822 through amino acid 829 of LDLR is shown in (SEQ ID NO: 62).

[00742] In another embodiment, the NPXY motif of LDLR may be modified to reduce the binding of Sorting Nexin 17 (SNX17) to the NPXY motif of LDLR. The reduction of binding of SNX17 to the NPXY motif of LDLR may be used to regulate the endosomal recycling of receptors.

[00743] In one embodiment, the PX domain (PI3P binding) of SNX17 may comprise at least one mutation. The at least one mutation may alter the ability of SNX17 to bind to the NPXY motif of LDLR and thus regulate the endosomal recycling of receptors.

[00744] In one embodiment, the FERM-like domain of SNX17 may comprise at least one mutation. The at least one mutation may alter the ability of SNX17 to bind to the NPXY motif of LDLR and thus regulate the endosomal recycling of receptors.

[00745] In one embodiment, the Ras-association domain of SNX17 may comprise at least one mutation. The at least one mutation may alter the ability of SNX17 to bind to the NPXY motif of LDLR and thus regulate the endosomal recycling of receptors.

[00746] In one embodiment, a LDLR sequence described herein may comprise at least one amino acid which has been phosphorylated. As a non-limiting example, at least one amino acid in the sequence NQDGYSYPSR (SEQ ID NO: 63) may be phosphorylated. As a non-limiting example, the two tyrosines (Ys) in SEQ ID NO: 63 of LDLR may be phosphorylated. As another non-limiting example, at least one tyrosine (Y) in the LDLR sequence described herein may be phosphorylated. As yet another non-limiting example, tyrosine at position 845 and tyrosine at position 847 of LDLR described herein are phosphorylated.

[00747] In one embodiment, a LDLR sequence described herein may comprise at least one amino acid which has been phosphorylated but tyrosine at position 828 is not phosphorylated.

[00748] In another embodiment, a LDLR sequences described herein may comprise at least one amino acid which has been phosphorylated, wherein at least one of the amino acids is tyrosine at position 828.

[00749] In one embodiment, the LDLR sequence described herein may comprise at least one amino acid mutation in the C-terminal sequence LEDDVA (SEQ ID NO: 64). As a non-limiting example, SEQ ID NO: 64 may be amino acid 855 through amino acid 860 of the LDLR sequence.

[00750] In one embodiment, the LDLR sequences may comprise at least one mutation at an N-linked glycosylation site of the LDLR sequence. As a non-limiting example, at least one mutation may be located at amino acid 97, 156, 272, 515 and/or 657.

[00751] In another embodiment, the LDLR sequences may comprise at least one mutation at an O-linked glycosylation site of the LDLR sequence. As a non-limiting example, at least one mutation may be located at amino acids 721-768.

[00752] In yet another embodiment, the LDLR sequences may comprise at least one mutation at an N-linked glycosylation site ant at least one mutation at an O-linked glycosylation site.

[00753] In one embodiment, the polynucleotides described herein may be deficient in binding to low density lipoprotein receptor adaptor protein 1 (LDLRAP1). While not wishing to be bound by theory, the LDLRAP1 binding-deficient LDLR may limit the binding and internalization of LDLR and thus reducing LDLR uptake.

[00754] In one embodiment, the ecto-domains of LDLR sequences and constructs described herein may be fused with cytoplasmic domains. As a non-limiting example, LDLR ecto-domain may be fused with folate receptor TM-cytoplasmic domain. As another non-limiting example, LDLR ecto-domain may be fused with GPI-linked receptor TM-cytoplasmic domain.

OTHER EMBODIMENTS

[00755] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview

of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[00756] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[00757] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

CLAIMS

1. A composition comprising;

(a) a synthetic polynucleotide encoding PCSK9 negative LDLR, and

(b) a synthetic polynucleotide encoding CYP7A1, in an acceptable diluent or carrier.

2. The composition of claim 1, further comprising;

(c) a statin selected from the group consisting of atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, simvastatin, and combinations thereof.

3. The composition of any of claims 1-2, wherein the synthetic polynucleotide comprises:

(a) a first region of linked nucleosides, said first region encoding at least one cholesterol regulating polypeptide;

(b) a first flanking region located at the 5' terminus of said first region comprising;

(i) a sequence of linked nucleosides selected from the group consisting of the native 5' untranslated region (UTR), SEQ ID NO: 1 and functional variants thereof;

(c) a second flanking region located at the 3' terminus of said first region comprising;

(i') a sequence of linked nucleosides selected from the group consisting of the native 3' UTR, any of the forgoing comprising one or more microRNA or microRNA binding site or microRNA seeds and functional variants or combinations thereof; and

(ii') a 3' tailing sequence of linked nucleosides;

wherein the first region of linked nucleosides comprises at least a first modified nucleoside.

4. The composition of claim 3 wherein the second flanking region encodes at least one miR binding site.

5. The composition of claim 4, wherein said miR binding site is selected from the group consisting of miR-122a and miR-422a.

6. The composition of any of claims 1-5 wherein the synthetic polynucleotide comprises at least one chemical modification.

7. A method comprising, contacting a hepatocyte in a subject with a composition of any of claims 1-6.

8. The method of claim 7 further comprising measuring cholesterol levels in plasma of said subject.

9. The method of claim 7, further comprising contacting said liver cell with a statin.

10. The method of claim 7 wherein the subject is a human.

11. The method of claim 10, wherein the human has a polymorphism in CYP7A1.

12. A method of treating a disease or disorder in a subject in need thereof comprising administering to said subject the composition of any of claims 1-6.

13. The method of claim 12 wherein the disease or disorder is selected from the group consisting of fatty liver disease, hepatocellular carcinoma, NASH, steatosis, familial hypercholesterolemia (FH), hypercholesterolemia, and aberrant lipoprotein profile.

14. The method of claim 12 wherein the disease or disorder is familiar hypercholesterolemia (FH).

15. The method of any of claims 7-14 wherein the composition further comprises a pharmaceutically acceptable excipient.

16. The method of any of claims 7-14, where the composition comprises a lipid and wherein said lipid is selected from DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200, DLin-MC3-DMA, reLNP, PLGA, PEG, PEG-DMA and PEGylated lipids and mixtures thereof.

17. The method of claim 12 wherein the synthetic polynucleotide is administered at a total daily dose of between 1 ug and 150 ug.

18. A method of modulating cholesterol levels in plasma of a subject comprising contacting said subject with the composition of any of claims 1-6.

19. The method of claims 18, wherein modulation is lowered levels.