[0005] Advancements in RNAi technology and delivery methodology have led to a growing number of positive outcomes with RNAi-based therapies. Such therapies represent a promising class of therapeutics, particularly against targets that have been deemed “undruggable” by small molecule or biologic modalities. Although much progress has been made to overcome the inherent metabolic liabilities of natural RNA through the development of chemical modifications and improved delivery methods, there remains a need in the art for RNAi agents with enhanced *in vivo* efficacy and stability suitable for administration for therapeutic purposes.

SUMMARY

[0006] The present disclosure is based, in part, on the design of chemical modification patterns for RNAi constructs that improve the potency and/or duration of gene silencing activity of the constructs *in vivo*. The modification patterns described herein can be universally applied to a variety of RNAi constructs having different sequences and targets. The RNAi constructs are useful for inhibiting target gene expression *in vivo,* for example for therapeutic purposes.

[0007] Accordingly, the present disclosure provides RNAi constructs that inhibit expression of a target gene sequence, wherein the RNAi constructs comprise a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, and wherein the RNAi constructs comprise a structure represented by one of the formulas described herein. In certain examples, the RNAi constructs of the disclosure have a chemical modification pattern selected from one of the patterns designated as P1 to P30 as described herein.

[0008] In some examples, the RNAi construct comprises a structure represented by Formula (A):

$$\begin{array}{l} 5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_M\ N_L\ N_M\ N_L\ N_T(n)_y-3' \\ 3'-(N_B)_z\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_M\ N_L\ N_M\ N_L\ N_L\ N_F\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5' \end{array}$$

(A)

[0009] In Formula (A), the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each N_F represents a 2′-fluoro

modified nucleotide; each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), and a deoxyribonucleotide; each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; and N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. X can be an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. One or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand. Y can be an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand. Z can be an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. One or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

[0010] In some examples, the RNAi construct comprises a sense strand of 19-23 nucleotides in length and an antisense strand of 19-23 nucleotides in length, wherein the sequences of the antisense stand and the sense strand are sufficiently complementary to each other to form a duplex region of 19-21 base pairs, wherein: nucleotides at positions 2, 7, and 14 in the antisense strand (counting from the 5′ end) are 2′-fluoro modified nucleotides; nucleotides in the sense strand at positions paired with positions 8 to 11 and 13 in the antisense strand (counting from the 5′ end) are 2′-fluoro modified nucleotides; and neither the sense strand nor the antisense strand each have more than 7 total 2′-fluoro modified nucleotides. The RNAi construct can have a

nucleotide overhang at one or both of the 3′ ends of the sense strand and the antisense strand. In certain examples, the RNAi construct has a nucleotide overhang at the 3′ end of the antisense strand and a blunt end at the 5′ end of the antisense strand.

[0011] In other examples of the disclosure, the RNAi construct comprises a structure represented by Formula (D):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_L\ N_M\ N_L\ N_L\ N_M\ N_M\ N_M\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5'$$

(D)

[0012] In Formula (D), the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each N_F represents a 2′-fluoro modified nucleotide; each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; and N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. X can be an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. One or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand. Y can be an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand. Z can be an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl

modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide. One or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

[0013] In some examples of the disclosure, the RNAi construct comprises a sense strand of 19-23 nucleotides in length and an antisense strand of 19-23 nucleotides in length, wherein the sequences of the antisense stand and the sense strand are sufficiently complementary to each other to form a duplex region of 19-21 base pairs, wherein: nucleotides at positions 2, 14, and 16 in the antisense strand (counting from the 5′ end) are 2′-fluoro modified nucleotides; nucleotides in the sense strand at positions paired with positions 10 to 13 in the antisense strand (counting from the 5′ end) are 2′-fluoro modified nucleotides; and neither the sense strand nor the antisense strand each have more than 7 total 2′-fluoro modified nucleotides. The RNAi construct can have a nucleotide overhang at the 3′ end of the antisense strand and a blunt end at the 5′ end of the antisense strand. Alternatively, the RNAi construct can have a nucleotide overhang at both of the 3′ ends of the sense strand and the antisense strand.

[0014] The RNAi constructs of the disclosure can comprise at least one backbone modification, such as a modified internucleotide or internucleoside linkage. In certain examples, the RNAi constructs described herein comprise at least one phosphorothioate internucleotide linkage. In particular examples, the phosphorothioate internucleotide linkages may be positioned at the 3′ or 5′ ends of the sense and/or antisense strands.

[0015] The RNAi constructs may further comprise a ligand to facilitate delivery or uptake of the RNAi constructs to specific tissues or cells, such as liver cells. In some examples, the ligand targets delivery of the RNAi constructs to hepatocytes. In these and other examples, the ligand may comprise galactose, galactosamine, or N-acetyl-galactosamine (GalNAc). In certain examples, the ligand comprises a multivalent galactose or multivalent GalNAc moiety, such as a trivalent or tetravalent galactose or GalNAc moiety. The ligand may be covalently attached to the 5′ or 3′ end of the sense strand of the RNAi construct, optionally through a linker. In some examples, the RNAi constructs comprise a ligand and linker having a structure according to any of Formulas I to IX described herein. In one example, the RNAi constructs comprise a ligand and linker having a structure according to Formula VI. In another example, the RNAi constructs comprise a ligand and linker having a structure according to Formula VII. In yet

another example, the RNAi constructs comprise a ligand and linker having a structure according to Formula IX.

[0016] The present disclosure also provides pharmaceutical compositions comprising any of the RNAi constructs described herein and a pharmaceutically acceptable carrier, excipient, or diluent. Such pharmaceutical compositions are particularly useful for reducing or inhibiting expression of a target gene in the cells (e.g. liver cells) of a subject, particularly when overexpression of the target gene product in the subject is associated with a pathological phenotype.

[0017] The present disclosure includes methods for reducing or inhibiting expression of a target gene in a cell, tissue, or subject. In one example, the methods comprise contacting the cell or tissue with any one of the RNAi constructs described herein. The cell or tissue may be *in vitro* or *in vivo*. In another example, the methods comprise administering any one of the RNAi constructs described herein to a subject. The RNAi constructs can be administered to the subject parenterally (e.g. intravenously or subcutaneously).

[0017a] In one aspect, the present disclosure refers to an RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (A):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_M\ N_L\ N_M\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_M\ N_L\ N_M\ N_L\ N_L\ N_F\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5'$$

(A)

wherein: the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each NF represents a 2′-fluoro modified nucleotide; each NM independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), and a deoxyribonucleotide; each NL independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a

deoxyribonucleotide; NT represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the NA nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the NA nucleotides can be complementary to nucleotides in the antisense strand; y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the NB nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the NB nucleotides can be complementary to NA nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

[0017b] In another aspect, the present disclosure refers to an RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (B):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_L\ N_L\ N_L\ N_L\ N_F\ N_F\ N_L\ N_F\ N_L\ N_F\ N_L-5'$$

(B)

wherein: the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each N_F represents a 2′-fluoro modified nucleotide; each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide,

a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand; y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

[0017c] In another aspect, the present disclosure refers to an RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (C):

$$5'\text{-}(Ab)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_M\ N_L\ N_M\ N_L\ N_T\text{-}3'$$
$$3'\text{-}N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_L\ N_M\ N_L\ N_F\ N_L\text{-}5'$$

(C)

wherein: the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each N_F represents a 2′-fluoro modified nucleotide; each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide,

a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, BNA, and a deoxyribonucleotide; N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; and x is 0 or 1 and Ab is an inverted abasic nucleotide.

[0017d] In another aspect, the present disclosure refers to an RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (D):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_L\ N_M\ N_L\ N_L\ N_M\ N_M\ N_M\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5'$$

(D)

wherein: the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand; each N_F represents a 2′-fluoro modified nucleotide; each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), and a deoxyribonucleotide; each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted

deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand; y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

[0017e] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the RNAi construct as disclosed herein and a pharmaceutically acceptable carrier or excipient.

[0017f] In another aspect, the present disclosure refers to an *in vitro* method for inhibiting the expression of a target gene in a cell comprising contacting the cell with the RNAi construct as disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1 shows several representative embodiments of chemical modification patterns for RNAi constructs. In each of the schematics, the top strand represents the sense strand in the 5′ to 3′ direction and the bottom strand represents the antisense strand in the 3′ to 5′ direction. Solid black circles represent 2′-O-methyl (2′-OMe) modified nucleotides, striped circles represent 2′-fluoro (2′-F) modified nucleotides, and white circles represent inverted abasic nucleotides (invAb) or inverted deoxyribonucleotides (invdN). Light gray lines connecting the circles represent phosphodiester linkages, whereas black lines connecting the circles represent phosphorothioate linkages. The black boxes denote the putative Ago2 cleavage sites within the RNAi constructs.

[0019] Figure 2 is a bar graph of human PNPLA3 variant expression levels in livers of mice injected with an AAV encoding the human PNPLA3 variant and treated with 5 mg/kg subcutaneous injections of the indicated RNAi construct having the P1 or CM1 chemical

modification pattern. Human PNPLA3 expression was measured by qPCR and is reported as expression levels relative to vehicle-treated animals. Expression levels are shown at day 8 after RNAi construct administration.

CLAIMS

What is claimed:

1. An RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (A):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_M\ N_L\ N_M\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_M\ N_L\ N_M\ N_L\ N_L\ N_F\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5'$$

(A)

wherein:

the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand;

each N_F represents a 2′-fluoro modified nucleotide;

each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), and a deoxyribonucleotide;

each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl

modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand;

y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and

z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

2. The RNAi construct of claim 1, wherein the sense strand and the antisense strand are each independently 19 to 30 nucleotides in length.

3. The RNAi construct of claim 1, wherein the sense strand and the antisense strand are each independently 19 to 25 nucleotides in length.

4. The RNAi construct of claim 1, wherein x is 0, y is 2, and z is 2.

5. The RNAi construct of claim 1, wherein x is 1 and N_A is an inverted abasic nucleotide, y is 2, and z is 2.

6. The RNAi construct of claim 1, wherein x is 2, y is 0, and z is 4.

7. The RNAi construct of claim 1, wherein x is 2, y is 0, and z is 2.

8. The RNAi construct of claim 1, wherein x is 3 and the N_A at the 5′ end is an inverted abasic nucleotide, y is 0, and z is 4.

9. The RNAi construct of claim 1, wherein x is 0, y is 0, and z is 2.

10. The RNAi construct of claim 1, wherein x is 1 and N_A is an inverted abasic nucleotide, y is 0, and z is 2.

11. The RNAi construct of any one of claims 1 to 10, wherein N_T is an inverted abasic nucleotide, an inverted deoxyribonucleotide, or a 2′-O-methyl modified nucleotide.

12. The RNAi construct of any one of claims 1 to 11, wherein each N_L in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

13. The RNAi construct of any one of claims 1 to 12, wherein the N_M at positions 4 and 12 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide.

14. The RNAi construct of claim 13, wherein the N_M at position 6 in the antisense strand counting from the 5′ end is a 2′-fluoro modified nucleotide.

15. The RNAi construct of claim 14, wherein the N_M at position 10 in the antisense strand counting from the 5′ end is a 2′-fluoro modified nucleotide.

16. The RNAi construct of any one of claims 1 to 12, wherein the N_M at positions 10 and 12 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide.

17. The RNAi construct of claim 16, wherein the N_M at position 4 in the antisense strand counting from the 5′ end is a 2′-fluoro modified nucleotide.

18. The RNAi construct of any one of claims 1 to 12, wherein the N_M at positions 4, 6, and 10 in the antisense strand counting from the 5′ end are each a 2′-O-methyl modified nucleotide, and the N_M at position 12 in the antisense strand counting from the 5′ end is a 2′-fluoro modified nucleotide.

19. The RNAi construct of any one of claims 1 to 12, wherein each N_M in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

20. The RNAi construct of any one of claims 1 to 18, wherein each N_M in the sense strand is a 2′-O-methyl modified nucleotide.

21. The RNAi construct of any one of claims 1 to 18, wherein each N_M in the sense strand is a 2′-fluoro modified nucleotide.

22. An RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (B):

$$\begin{array}{l} 5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_T(n)_y-3' \\ 3'-(N_B)_z\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_L\ N_L\ N_L\ N_L\ N_F\ N_F\ N_L\ N_F\ N_L\ N_F\ N_L-5' \end{array}$$

(B)

wherein:

the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand;

each N_F represents a 2′-fluoro modified nucleotide;

each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl

modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand;

y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and

z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

23. The RNAi construct of claim 22, wherein x is 0, y is 2, and z is 2.

24. The RNAi construct of claim 22, wherein x is 0, y is 0, and z is 2.

25. The RNAi construct of claim 22, wherein x is 1 and N_A is an inverted abasic nucleotide, y is 2, and z is 2.

26. The RNAi construct of claim 22, wherein x is 2, y is 0, and z is 4.

27. The RNAi construct of claim 22, wherein x is 3 and the N_A at the 5′ end is an inverted abasic nucleotide, y is 0, and z is 4.

28. The RNAi construct of any one of claims 22 to 27, wherein N_T is an inverted abasic nucleotide, an inverted deoxyribonucleotide, or a 2′-O-methyl modified nucleotide.

29. The RNAi construct of any one of claims 22 to 28, wherein each N_L in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

30. An RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (C):

$$5'\text{-}(Ab)_x\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_M\ N_L\ N_M\ N_L\ N_T\text{-}3'$$
$$3'\text{-}N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_F\ N_L\ N_L\ N_L\ N_L\ N_F\ N_L\ N_L\ N_M\ N_L\ N_F\ N_L\text{-}5'$$

(C)

wherein:

the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand;

each N_F represents a 2′-fluoro modified nucleotide;

each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, BNA, and a deoxyribonucleotide;

N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide; and

x is 0 or 1 and Ab is an inverted abasic nucleotide.

31. The RNAi construct of claim 30, wherein each N_M in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

32. The RNAi construct of claim 31, wherein N_T is an inverted abasic nucleotide or inverted deoxyribonucleotide and x is 0.

33. The RNAi construct of claim 31, wherein N_T is a 2′-O-methyl modified nucleotide and x is 1.

34. The RNAi construct of claim 30, wherein the N_M in the antisense strand is a 2′-fluoro modified nucleotide.

35. The RNAi construct of claim 34, wherein each N_M in the sense strand is a 2′-O-methyl modified nucleotide.

36. The RNAi construct of claim 34, wherein each N_M in the sense strand is a 2′-fluoro modified nucleotide.

37. The RNAi construct of any one of claims 34 to 36, wherein N_T is an inverted abasic nucleotide or inverted deoxyribonucleotide and x is 0.

38. The RNAi construct of any one of claims 30 to 37, wherein each N_L in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

39. An RNAi construct that inhibits expression of a target gene sequence, comprising a sense strand and an antisense strand, wherein the antisense strand comprises a sequence that is complementary to the target gene sequence and the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region, wherein the RNAi construct comprises a structure represented by Formula (D):

$$5'-(N_A)_x\ N_L\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_F\ N_F\ N_F\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_L\ N_T(n)_y-3'$$
$$3'-(N_B)_z\ N_L\ N_L\ N_L\ N_M\ N_L\ N_F\ N_L\ N_M\ N_L\ N_L\ N_M\ N_M\ N_M\ N_M\ N_L\ N_M\ N_L\ N_F\ N_L-5'$$

(D)

wherein:

the top strand listed in the 5′ to 3′ direction is the sense strand and the bottom strand listed in the 3′ to 5′ direction is the antisense strand;

each N_F represents a 2′-fluoro modified nucleotide;

each N_M independently represents a modified nucleotide selected from a 2′-fluoro modified nucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), and a deoxyribonucleotide;

each N_L independently represents a modified nucleotide selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

N_T represents a modified nucleotide selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide;

x is an integer from 0 to 4, provided that when x is 1, 2, 3, or 4, one or more of the N_A nucleotides is a modified nucleotide independently selected from an abasic nucleotide, an inverted abasic nucleotide, an inverted deoxyribonucleotide, a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_A nucleotides can be complementary to nucleotides in the antisense strand;

y is an integer from 0 to 4, provided that when y is 1, 2, 3, or 4, one or more n nucleotides are modified or unmodified overhang nucleotides that do not base pair with nucleotides in the antisense strand; and

z is an integer from 0 to 4, provided that when z is 1, 2, 3, or 4, one or more of the N_B nucleotides is a modified nucleotide independently selected from a 2′-O-methyl modified nucleotide, a 2′-O-methoxyethyl modified nucleotide, a 2′-O-alkyl modified nucleotide, a 2′-O-allyl modified nucleotide, a BNA, and a deoxyribonucleotide, and one or more of the N_B nucleotides can be complementary to N_A nucleotides when present in the sense strand or can be overhang nucleotides that do not base pair with nucleotides in the sense strand.

40. The RNAi construct of claim 39, wherein the sense strand and the antisense strand are each independently 19 to 30 nucleotides in length.

41. The RNAi construct of claim 39, wherein the sense strand and the antisense strand are each independently 19 to 25 nucleotides in length.

42. The RNAi construct of claim 39, wherein x is 2, y is 0, and z is 4.

43. The RNAi construct of claim 39, wherein x is 1 and N_A is an inverted abasic nucleotide, y is 2, and z is 2.

44. The RNAi construct of claim 39, wherein x is 1 and N_A is an inverted abasic nucleotide, y is 0, and z is 2.

45. The RNAi construct of claim 39, wherein x is 0, y is 0, and z is 2.

46. The RNAi construct of claim 39, wherein x is 2, y is 0, and z is 2.

47. The RNAi construct of any one of claims 39 to 46, wherein N_T is an inverted abasic nucleotide, an inverted deoxyribonucleotide, or a 2′-O-methyl modified nucleotide.

48. The RNAi construct of any one of claims 39 to 47, wherein each N_L in both the sense and antisense strands is a 2′-O-methyl modified nucleotide.

49. The RNAi construct of any one of claims 39 to 48, wherein the N_M at positions 4, 6, 8, 9, and 16 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide and the N_M at positions 7 and 12 in the antisense strand counting from the 5′ end are each a 2′-O-methyl modified nucleotide.

50. The RNAi construct of any one of claims 39 to 48, wherein the N_M at positions 4, 6, 8, 9, and 16 in the antisense strand counting from the 5′ end are each a 2′-O-methyl modified nucleotide and the N_M at positions 7 and 12 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide.

51. The RNAi construct of any one of claims 39 to 48, wherein the N_M at positions 4, 6, 8, 9, and 12 in the antisense strand counting from the 5′ end are each a 2′-O-methyl modified nucleotide and the N_M at positions 7 and 16 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide.

52. The RNAi construct of any one of claims 39 to 48, wherein the N_M at positions 7, 8, 9, and 12 in the antisense strand counting from the 5′ end are each a 2′-O-methyl modified nucleotide and the N_M at positions 4, 6, and 16 in the antisense strand counting from the 5′ end are each a 2′-fluoro modified nucleotide.

53. The RNAi construct of any one of claims 39 to 52, wherein the N_M in the sense strand is a 2′-fluoro modified nucleotide.

54. The RNAi construct of any one of claims 39 to 52, wherein the N_M in the sense strand is a 2′-O-methyl modified nucleotide.

55. The RNAi construct of any one of claims 1 to 54, wherein the sense strand, the antisense strand, or both the sense and antisense strands comprise one or more phosphorothioate internucleotide linkages.

56. The RNAi construct of claim 55, wherein the antisense strand comprises two consecutive phosphorothioate internucleotide linkages between the terminal nucleotides at both the 3′ and 5′ ends.

57. The RNAi construct of claim 55 or 56, wherein the sense strand comprises a single phosphorothioate internucleotide linkage between the terminal nucleotides at the 3′ end.

58. The RNAi construct of claim 55 or 56, wherein the sense strand comprises two consecutive phosphorothioate internucleotide linkages between the terminal nucleotides at the 3′ end.

59. The RNAi construct of any one of claims 1 to 58, wherein the RNAi construct further comprises a ligand.

60. The RNAi construct of claim 59, wherein the ligand comprises a cholesterol moiety, a vitamin, a steroid, a bile acid, a folate moiety, a fatty acid, a carbohydrate, a glycoside, or antibody or antigen-binding fragment thereof.

61. The RNAi construct of claim 59, wherein the ligand targets delivery of the RNAi construct to hepatocytes.

62. The RNAi construct of claim 59, wherein the ligand comprises galactose, galactosamine, or N-acetyl-galactosamine.

63. The RNAi construct of claim 62, wherein the ligand comprises a multivalent galactose moiety or multivalent N-acetyl-galactosamine moiety.

64. The RNAi construct of claim 63, wherein the multivalent galactose moiety or multivalent N-acetyl-galactosamine moiety is trivalent or tetravalent.

65. The RNAi construct of any one of claims 59 to 64, wherein the ligand is covalently attached to the sense strand optionally through a linker.

66. The RNAi construct of claim 65, wherein the ligand is covalently attached to the 5′ end of the sense strand.

67. A pharmaceutical composition comprising the RNAi construct of any one of claims 1 to 66 and a pharmaceutically acceptable carrier or excipient.

68. An *in vitro* method for inhibiting the expression of a target gene in a cell comprising contacting the cell with the RNAi construct of any one of claims 1 to 66.

69. The RNAi construct of any one of claims 1 to 66 for use in therapy.

70. The RNAi construct of claim 69, wherein the RNAi construct is formulated for a parenteral route of administration.