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COMPOSITIONS AND METHODS FOR TREATING LIVER DISEASES WITH siRNAS TARGETING TBX3

Abstract

Disclosed herein are compositions comprising siRNAs capable of downregulating T-box transcription factor 3 (TBX3) gene expression or a variant thereof. Also disclosed herein are methods of using such compositions in the treatment of a liver disease or injury, such as fatty liver disease (FLD), non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH).

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Background/Summary

CROSS REFERENCE TO RELATED APPLICATIONS [0001] This application claims the benefit of U.S. Provisional Application No. 63/328,549, filed Apr. 7, 2022, and U.S. Provisional Application No. 63/491,264, filed Mar. 20, 2023, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

SEQUENCE LISTING
[0002] This application contains a Sequence Listing that has been submitted in xml format via EFS-Web and is hereby incorporated by reference in its entirety. The xml copy, created on Apr. 7, 2023, is named “106546-755473.xml” and is about 281.4 KB in size.
BACKGROUND
1. Field
[0003] The present inventive concept is directed to compositions and methods of use thereof for liver disease treatment, specifically to metabolic liver diseases, including but not limited to non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH).
2. Discussion of Related Art
[0004] NAFLD is a spectrum of chronic liver disorders, which encompass, among others nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NASH, a common cause of chronic liver disease, is defined as having at least 5% hepatic steatosis and inflammation with or without fibrosis. Over time and without treatment, NASH may progress to cirrhosis and even hepatocellular carcinoma (HCC). Moreover, NAFLD/NASH is strongly associated with obesity and type II diabetes which together affect over 50% of the US population, leading to a heavy economic burden. Unfortunately, therapeutic options for NASH remain limited, with only slight benefits observed from vitamin E or obeticholic acid treatment. While NASH is the leading cause of chronic liver disease and cirrhosis, there are currently no clinically approved therapies. As such, new targets, therapeutics, and combinations thereof are needed to accelerate clinical progress in the treatment of liver diseases.
SUMMARY OF THE INVENTION
[0005] The present disclosure is based, in part, on the novel finding that positive selection of somatic mutations in NASH patient livers can increase cell fitness and competitiveness through decreased lipid accumulation in NASH. In vivo genetic screening for transcriptional regulators that promote clonal fitness through the reversal of lipotoxicity identified TBX3 (T-box transcription factor 3). Accordingly, the present disclosure provides for novel compositions for modulating TBX3 expression and methods of preventing, attenuating and/or treating liver diseases.
[0006] In some aspects, the current disclosure encompasses a composition comprising a nucleic acid that downregulates expression of TBX3 (T-box transcription factor 3) or a variant thereof. In some aspects, the nucleic acid that downregulates expression of TBX3 comprises a siRNA, a cluster regularly interspaced short palindromic repeats (CRISPR) related nucleic acid, a single guide RNA (sgRNA), a CRISPR-RNA (crRNA), or a trans-activating crRNA (tracrRNA). In some aspects, the nucleic acid is a small interfering RNA (siRNA) molecule. In some aspects, the composition comprises a plasmid or a viral vector, wherein the plasmid or the viral vector comprises a nucleic acid encoding the siRNA molecule as disclosed herein. In some aspects, the siRNA molecule comprises a nucleotide sequence that is 2 to 30 nucleotides in length and is at least 80% homologous to at least 2 to 30 contiguous nucleotides of a human TBX3 cDNA sequence. In

some aspects, the human TBX3 cDNA sequence comprises SEQ ID NO: 1. In some aspects the siRNA molecule targets the open reading frame or the 5′ or 3′ UTRs of the TBX3 gene. In some aspects, the siRNA molecule comprises at least one sense sequence, at least one antisense sequence, or at least one sense sequence and at least one antisense sequence. In some aspects, the siRNA molecule comprises a nucleotide sequence SEQ ID NOs: 2-115 or any combination thereof. In some aspects, the at least one sense sequence comprises SEQ ID NOs: 2-58. In some aspects, the at least one antisense sequence comprises SEQ ID NOs: 59-115.

[0007] In some aspects, the current disclosure also encompasses a composition comprising a nucleic acid molecule that downregulates expression of TBX3, wherein the nucleic acid is a sgRNA or encodes an sgRNA. In some aspects, the composition comprises a plasmid or a viral vector, wherein the plasmid or the viral vector comprises a first nucleic acid encoding the sgRNA molecule as disclosed herein and optionally a second nucleic acid encoding an RNA guided nuclease. In some aspects, the sgRNA target sequence is at least about 80% identical to any one of SEQ ID NO: 118-217. In some aspects, the RNA guided nuclease is a Cas endonuclease.

[0008] In some aspects, the siRNA molecule as disclosed herein specifically downregulates gene expression of at least one variant of TBX3. In some aspects, the sgRNA molecule specifically downregulates gene expression of at least one variant of TBX3. In some aspects, the at least one variant of GPAM is associated with a liver disease. Non-limiting examples of liver disease comprises fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer, or any combination thereof.

[0009] In some aspects, the nucleic acid molecule as disclosed herein may be conjugated to least one targeting ligand. In some aspects, at least one targeting ligand comprises a liver targeting ligand. In some aspects, the liver targeting ligand comprises at least one N-acetylgalactosamine (GalNAc) conjugate. In some aspects, the nucleic acid molecule is conjugated to about one to about three GalNAc conjugates. In some aspects, the nucleic acid molecule comprises at least one chemical modification. In some aspects, the nucleic acid molecule comprises a modification at least one ribosugar moiety of its nucleotide sequence. In some aspects, the at least one ribosugar moiety is modified with 2 2′-O-methyl (2′OMe), 2′-deoxy-2′-fluoro (2′F), 2′-deoxy, 5-C-methyl, 2′-O-(2-methoxyethyl) (MOE), 4′-thio, 2′-amino, 2′-C-allyl, or any combination thereof. In some aspects, less than about 10% to about 70% of ribosugar moieties of the total nucleotide sequence is modified.

[0010] In some aspects, the current disclosure also encompasses a pharmaceutical composition comprising any one of the compositions as disclosed herein and at least one pharmaceutically acceptable carrier. In some aspects, the pharmaceutical composition further comprises a nanoparticle. In some aspects, the pharmaceutical composition further comprises a lipid.

[0011] In some aspects, the current disclosure also encompasses a method of treating a subject in need thereof, the method comprising administrating a therapeutically effective amount of the composition as disclosed herein, or the pharmaceutical composition as disclosed herein. In some aspects, the subject in need thereof, is a human subject having or suspected of having a liver disease. In some aspects, the liver disease comprises fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer, or any combination thereof. In some aspects, the method of administering comprises parenteral administration. In some aspects, the administration of a therapeutically effective amount of the composition as disclosed here or the pharmaceutical composition as disclosed herein increases life expectancy of the subject compared to an untreated subject with identical disease condition and predicted outcome. In some aspects of the method, administration of a therapeutically effective amount of the composition as disclosed herein or the pharmaceutical composition as disclosed herein increases liver function of the subject compared to an untreated subject with identical disease condition and predicted outcome. In some

aspects of the method, administration of a therapeutically effective amount of the composition as disclosed herein or the pharmaceutical composition as disclosed herein attenuates liver fibrosis in the subject compared to an untreated subject with identical disease condition and predicted outcome. In some aspects, the administration of a therapeutically effective amount of the composition as disclosed herein or the pharmaceutical composition as disclosed herein prevents additional liver fibrosis in the subject compared to an untreated subject with identical disease condition and predicted outcome.

[0012] In some aspects, the current disclosure also encompasses a kit comprising: a container holding the composition as disclosed here or the pharmaceutical composition as disclosed herein, a pharmaceutical administrative means; and instructions for use.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to the drawing in combination with the detailed description of specific embodiments presented herein. Embodiments of the present inventive concept are illustrated by way of example in which like reference numerals indicate similar elements and in which:

[0014] FIG. **1** depicts a conditional liver gene deletion analysis using CRISPR, showing Tbx3 gene knockout resulting in reduced liver to body weight percentages. Single guide RNAs targeting GFP or LacZ delivered into mice with Cas9 expression served as controls and single guide RNAs targeting Tbx3 delivered into mice with Cas9 expression served as liver specific models of Tbx3 deletion. All mice were fed with western diets and high sugar water for 3 months to induce a NASH phenotype, and each mouse is shown in FIG. **1** as a single red or blue dot. There was a statistically significant reduction in liver weight to body weight percentage after Tbx3 deletion, indicating protection from NASH.

[0015] FIG. **2**A shows the liver histology after three (3) months of NASH diets in GFP knockout mice at 5×.

[0016] FIG. **2**B shows the liver histology after three (3) months of NASH diets in GFP knockout mice at 15×.

[0017] FIG. **2**C shows the liver histology after three (3) months of NASH diets in LacZ knockout mice at 5×.

[0018] FIG. **2**D shows the liver histology after three (3) months of NASH diets in LacZ knockout mice at 15×.

[0019] FIG. **2**E shows the liver histology after three (3) months of NASH diets in Tbx3 CRISPR knockout mice at 5×.

[0020] FIG. **2**F shows the liver histology after three (3) months of NASH diets in Tbx3 CRISPR knockout mice at 15×. Representative images show significantly reduced hepatic steatosis in Tbx3 CRISPR knockout mice compared to the GFP and LacZ groups, which were representative controls.

[0021] FIG. **3**A shows a luciferase-based screen used to test the relative knockdown efficacy for various TBX3 siRNAs.

[0022] FIG. **3**B shows representative images of TBX3 protein levels examined using western blot analysis after siRNA knockdown.

[0023] FIG. **4**A shows body weight ratios of control (sgGFP and sgLacZ) and liver-specific KO mice fed with 3 months of WD. Gray dots represent control mice, blue dots represent liver-specific KO mice for known NASH genes, red dots represent transcription factor KO mice, and green dots represent epigenetic factor KO mice. Darker dots represent mice that have the most significant

differences in liver/body weight ratios. Each dot represents one mouse, and the n is denoted at the bottom of each plot.
[0024] FIG. **4**B shows liver/body weight ratios of control (sgGFP and sgLacZ) and liver-specific KO mice fed with 3 months of WD. Gray dots represent control mice, blue dots represent liver-specific KO mice for known NASH genes, red dots represent transcription factor KO mice, and green dots represent epigenetic factor KO mice. Darker dots represent mice that have the most significant differences in liver/body weight ratios. Each dot represents one mouse, and the n is denoted at the bottom of each plot.
[0025] FIG. **4**C shows liver function testing using plasma ALT. Gray dots represent control mice, blue dots represent liver-specific KO mice for known NASH genes, red dots represent transcription factor KO mice, and green dots represent epigenetic factor KO mice. Darker dots represent mice that have the most significant differences in liver/body weight ratios. Each dot represents one mouse, and the n is denoted at the bottom of each plot.
[0026] FIG. **4**D shows liver function testing using plasma AST.
[0027] FIG. **4**E shows liver function testing using liver triglycerides.
[0028] FIG. **4**F shows liver function testing using liver cholesterol.
[0029] FIG. **4**G shows representative H&E images of liver section are shown.
[0030] FIG. **4**H shows H&E staining of liver sections from transcription factor KO mice.
[0031] FIG. **4**I shows H&E staining of liver sections from epigenetic factor KO mice.
[0032] FIG. **5**A shows body weight, liver weight, and liver/body weight ratios of male control, Tbx3 KO and Smyd2 KO mice fed with 9 months of WD (n=7, 13, 8 mice for each group).
[0033] FIG. **5**B shows liver function testing with plasma ALT and AST for the mice (n=7, 12, 8 mice for each group).
[0034] FIG. **5**C shows plasma cholesterol and triglycerides for the mice (n=7, 12, 8 mice for each group).
[0035] FIG. **5**D shows H&E and Sirius Red staining of liver sections for the mice.
[0036] FIG. **5**E shows quantification of Sirius Red staining images in **5**D. Each dot represents one image field; three fields from each mouse liver were analyzed. Statistical analysis was performed on averaged image data from individual mice.
[0037] FIG. **6**A shows a Venn diagram showing the shared and unique gene numbers with changed expression (fold change >=1.5) in Bcl6, Tbx3, and Irs1 KO livers.
[0038] FIG. **6**B shows a hallmark pathway enrichment analysis of RNA-seq data.
[0039] FIG. **6**C shows heatmaps showing the fold changes of differentially expressed genes in fatty acid, triglyceride, and cholesterol synthesis pathways. The average expression levels of control samples (four sgGFP and two sgLacZ) were normalized to 1 for each gene.
[0040] FIG. **6**D shows heatmaps showing the fold changes of differentially expressed collagen genes. The average expression levels of control samples (four sgGFP and two sgLacZ) were normalized to 1 for each gene.
[0041] The drawing figures do not limit the present inventive concept to the specific embodiments disclosed and described herein. The drawings are not necessarily to scale, emphasis instead being placed on clearly illustrating principles of certain embodiments of the present inventive concept.

DETAILED DESCRIPTION

[0042] The following detailed description references the accompanying drawings that illustrate various embodiments of the present inventive concept. The drawings and description are intended to describe aspects and embodiments of the present inventive concept in sufficient detail to enable those skilled in the art to practice the present inventive concept. Other components can be utilized and changes can be made without departing from the scope of the present inventive concept. The following description is, therefore, not to be taken in a limiting sense. The scope of the present inventive concept is defined only by the appended claims, along with the full scope of equivalents to which such claims are entitled.

[0043] With increasing over-nutrition and obesity, non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the leading cause of liver disease in the world. NAFLD is usually conceptualized at the organismal and tissue levels; however, little thought has been given to genetic heterogeneity within clones of the liver. Somatic mutations are common in most healthy individuals, and there is accumulating evidence that mutation burden increases with age and chronic tissue damage. The present disclosure is based, in part, on the novel finding that positive selection of somatic mutations in NASH patient livers can increase cell fitness and competitiveness through decreased lipid accumulation in NASH. Based on this novel concept, in vivo genetic screening of somatically mutated transcriptional regulators dysregulated in human liver disease was performed. This genetic screen identified genes that when mutated, promoted clonal fitness through the reversal of lipotoxicity, including TBX3 (T-box transcription factor 3). Accordingly, provided herein are compositions and methods of use thereof for liver disease treatment, specifically to metabolic liver diseases, including but not limited to non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH).

I. Terminology

[0044] The phraseology and terminology employed herein are for the purpose of description and should not be regarded as limiting. For example, the use of a singular term, such as, “a” is not intended as limiting of the number of items. Also, the use of relational terms such as, but not limited to, “top,” “bottom,” “left,” “right,” “upper,” “lower,” “down,” “up,” and “side,” are used in the description for clarity in specific reference to the figures and are not intended to limit the scope of the present inventive concept or the appended claims.

[0045] Further, as the present inventive concept is susceptible to embodiments of many different forms, it is intended that the present disclosure be considered as an example of the principles of the present inventive concept and not intended to limit the present inventive concept to the specific embodiments shown and described. Any one of the features of the present inventive concept may be used separately or in combination with any other feature. References to the terms “embodiment,” “embodiments,” and/or the like in the description mean that the feature and/or features being referred to are included in, at least, one aspect of the description. Separate references to the terms “embodiment,” “embodiments,” and/or the like in the description do not necessarily refer to the same embodiment and are also not mutually exclusive unless so stated and/or except as will be readily apparent to those skilled in the art from the description. For example, a feature, structure, process, step, action, or the like described in one embodiment may also be included in other embodiments but is not necessarily included. Thus, the present inventive concept may include a variety of combinations and/or integrations of the embodiments described herein. Additionally, all aspects of the present disclosure, as described herein, are not essential for its practice. Likewise, other systems, methods, features, and advantages of the present inventive concept will be, or become, apparent to one with skill in the art upon examination of the figures and the description. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present inventive concept, and be encompassed by the claims.

[0046] As used herein, the term “about,” can mean relative to the recited value, e.g., amount, dose, temperature, time, percentage, etc., ±10%, ±9%, ±8%, ±7%, ±6%, ±5%, ±4%, ±3%, ±2%, or ±1%.

[0047] The terms “comprising,” “including,” “encompassing” and “having” are used interchangeably in this disclosure. The terms “comprising,” “including,” “encompassing” and “having” mean to include, but not necessarily be limited to the things so described.

[0048] The terms “or” and “and/or,” as used herein, are to be interpreted as inclusive or meaning any one or any combination. Therefore, “A, B or C” or “A, B and/or C” mean any of the following: “A,” “B” or “C”; “A and B”; “A and C”; “B and C”; “A, B and C.” An exception to this definition will occur only when a combination of elements, functions, steps or acts are in some way inherently mutually exclusive.

[0049] “Biomarker” as used herein refers to any biological molecules (e.g., nucleic acids, genes, peptides, proteins, lipids, hormones, metabolites, and the like) that, singularly or collectively, reflect the current or predict future state of a biological system. Thus, as used herein, the presence or concentration of one or more biomarkers can be detected and correlated with a known condition, such as a disease state. In some aspects, detecting the presence and/or concentration of one or more biomarkers herein may be an indication of a liver disease risk in a subject. In some other aspects, detecting the presence and/or concentration of one or more biomarkers herein may be used in treating and/or preventing a chronic liver disease in a subject.

[0050] As used herein, the terms “treat”, “treating”, “treatment” and the like, unless otherwise indicated, can refer to reversing, alleviating, inhibiting the process of, or preventing the disease, disorder or condition to which such term applies, or one or more symptoms of such disease, disorder or condition and includes the administration of any of the compositions, pharmaceutical compositions, or dosage forms described herein, to prevent the onset of the symptoms or the complications, or alleviating the symptoms or the complications, or eliminating the condition, or disorder.

[0051] The term “biomolecule” as used herein refers to, but is not limited to, proteins, enzymes, antibodies, DNA, siRNA, and small molecules. “Small molecules” as used herein can refer to chemicals, compounds, drugs, and the like.

[0052] The term “nucleic acid” or “polynucleotide” refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

[0053] The terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. A polypeptide includes a natural peptide, a recombinant peptide, or a combination thereof.

[0054] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

II. Compositions

(a) TBX3

[0055] In certain embodiments, compositions for use in the methods disclosed herein can modulate TBX3 (T-box transcription factor 3) gene. The TBX3 gene is a member of a phylogenetically conserved family of genes that share a common DNA-binding domain, the T-box. These genes

encode transcription factors involved in the regulation of developmental processes. Additionally, alternative splicing results in three transcript variants encoding different isoforms.

[0056] As used herein, compositions “modulating” TBX3 can include any biomolecule(s) capable of decreasing TBX3 gene expression, decreasing TBX3 protein expression, decreasing TBX3 activity, or a combination thereof. In some aspects, biomolecule(s) herein capable of modulating TBX3 can be an inhibitor of TBX3. As used herein, an inhibitor of TBX3 can inhibit TBX3 direct activity, inhibit TBX3 indirect activity, decrease expression of the TBX3 gene, decrease expression of the TBX3 protein, or a combination thereof.

[0057] In certain embodiments, compositions for use in the methods disclosed herein can include a nucleic acid molecule. The term “nucleic acid molecule” as used herein refers to a molecule having nucleotides. The nucleic acid can be single, double, or multiple stranded and may comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof. In some embodiments, a nucleic acid molecule for use herein can be a double-stranded RNA. In some examples, a double stranded RNA suitable for use herein can be small temporal RNA, small nuclear RNA, small nucleolar RNA, short hairpin RNA, microRNA, or the like. In certain embodiments, a double stranded RNA suitable for use herein can be a small interfering RNA (siRNA).

[0058] The term “siRNA” as used herein refers to small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway. A siRNA molecule disclosed herein may be capable of silencing, reducing, and/or inhibiting expression of a target gene (e.g., TBX3). These molecules can vary in length (generally about 5-50 base pairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNA have unpaired overhanging bases on the 5′ or 3′ end of the sense strand and/or the antisense strand. The term “siRNA” as used herein can include duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region.

[0059] siRNA molecules disclosed herein may be any interfering RNA with a duplex length of about 2-60, about 5-50, or about 10-40 nucleotides in length, more typically about 2-30, about 5-25, or about 10-25 nucleotides in length. In some embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 2 to about 30 nucleotides in length, about 5 to about 25 nucleotides in length, or about 10 to about 20 nucleotides in length. In some embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 10 nucleotides in length, about 11 nucleotides in length, about 12 nucleotides in length, about 13 nucleotides in length, about 14 nucleotides in length, about 15 nucleotides in length, about 14 nucleotides in length, about 15 nucleotides in length, about 16 nucleotides in length, about 17 nucleotides in length, about 18 nucleotides in length, about 19 nucleotides in length, about 20 nucleotides in length, about 21 nucleotides in length, about 22 nucleotides in length, about 23 nucleotides in length, about 24 nucleotides in length, or about 25 nucleotides in length. Each complementary sequence of a double-stranded siRNA disclosed herein may be about 2-60, about 5-50, about 10-40, about 2-30, about 5-25, or about 10-25 nucleotides in length, but other noncomplementary sequences may be present. For example, siRNA duplexes may comprise 3′ overhangs of about 1 to about 4 or more nucleotides and/or 5′ phosphate termini comprising about 1 to about 4 or more nucleotides.

[0060] In certain embodiments, siRNA molecules disclosed herein may have about 2-60, about 2-50, about 2-40, or about 2-30 contiguous nucleotides of homology with a target (e.g., TBX3) nucleotide sequence. In some aspects, a target nucleotide sequence herein may be a human TBX3 nucleotide sequence or a variant thereof. In some other aspects, a target nucleotide sequence herein may be human TBX3, RefSeq: NM_005996.4, or a variant thereof. In still some other aspects, a target nucleotide sequence herein may be SEQ ID NO: 1 as follows, or a variant thereof:

TABLE-US-00001 Human TBX3 (SEQ ID NO: 1):
AGCCTCTCCATGAGAGATCCGGTCATTCCTGGGACAAGCATGGCCTACCATCCGTTCCT

ACCTCACCGGGCGCCGGACTTCGCCATGAGCGCGGTGCTGGGTCACCAGCCGCCGTTCT
TCCCCGCGCTGACGCTGCCTCCCAACGGCGCGGCGGCGCTCTCGCTGCCGGGCGCCCTG
GCCAAGCCGATCATGGATCAATTGGTGGGGGCGGCCGAGACCGGCATCCCGTTCTCCTC
CCTGGGGCCCCAGGCGCATCTGAGGCCTTTGAAGACCATGGAGCCCGAAGAAGAGGTGG
AGGACGACCCCAAGGTGCACCTGGAGGCTAAAGAACTTTGGGATCAGTTTCACAAGCGG
GGCACCGAGATGGTCATTACCAAGTCGGGAAGGCGAATGTTTCCTCCATTTAAAGTGAG
ATGTTCTGGGCTGGATAAAAAAGCCAAATACATTTTATTGATGGACATTATAGCTGCTG
ATGACTGTCGTTATAAATTTCACAATTCTCGGTGGATGGTGGCTGGTAAGGCCGACCCC
GAAATGCCAAAGAGGATGTACATTCACCCGGACAGCCCCGCTACTGGGGAACAGTGGAT
GTCCAAAGTCGTCACTTTCCACAAACTGAAACTCACCAACAACATTTCAGACAAACATG
GATTTACTTTGGCCTTCCCAAGTGATCACGCTACGTGGCAGGGGAATTATAGTTTTGGT
ACTCAGACTATATTGAACTCCATGCACAAATACCAGCCCCGGTTCCACATTGTAAGAGC
CAATGACATCTTGAAACTCCCTTATAGTACATTTCGGACATACTTGTTCCCCGAAACTG
AATTCATCGCTGTGACTGCATACCAGAATGATAAGATAACCCAGTTAAAAATAGACAAC
AACCCTTTTGCAAAAGGTTTCCGGGACACTGGAAATGGCCGAAGAGAAAAAAGAAAACA
GCTCACCCTGCAGTCCATGAGGGTGTTTGATGAAAGACACAAAAAGGAGAATGGGACCT
CTGATGAGTCCTCCAGTGAACAAGCAGCTTTCAACTGCTTCGCCCAGGCTTCTTCTCCA
GCCGCCTCCACTGTAGGGACATCGAACCTCAAAGATTTATGTCCCAGCGAGGGTGAGAG
CGACGCCGAGGCCGAGAGCAAAGAGGAGCATGGCCCCGAGGCCTGCGACGCGGCCAAGA
TCTCCACCACCACGTCGGAGGAGCCCTGCCGTGACAAGGGCAGCCCCGCGGTCAAGGCT
CACCTTTTCGCTGCTGAGCGGCCCCGGGACAGCGGGCGGCTGGACAAAGCGTCGCCCGA
CTCACGCCATAGCCCCGCCACCATCTCGTCCAGCACTCGCGGCCTGGGCGCGGAGGAGC
GCAGGAGCCCGGTTCGCGAGGGCACAGCGCCGGCCAAGGTGGAAGAGGCGCGCGCGCTC
CCGGGCAAGGAGGCCTTCGCGCCGCTCACGGTGCAGACGGACGCGGCCGCCGCGCACCT
GGCCCAGGGCCCCCTGCCTGGCCTCGGCTTCGCCCCGGGCCTGGCGGGCCAACAGTTCT
TCAACGGGCACCCGCTCTTCCTGCACCCCAGCCAGTTTGCCATGGGGGGCGCCTTCTCC
AGCATGGCGGCCGCTGGCATGGGTCCCCTCCTGGCCACGGTTTCTGGGGCCTCCACCGG
TGTCTCGGGCCTGGATTCCACGGCCATGGCCTCTGCCGCTGCGGCGCAGGGACTGTCCG
GGGCGTCCGCGGCCACCCTGCCCTTCCACCTCCAGCAGCACGTCCTGGCCTCTCAGGGC
CTGGCCATGTCCCCTTTCGGAAGCCTGTTCCCTTACCCCTACACGTACATGGCCGCAGC
GGCGGCCGCCTCCTCTGCGGCAGCCTCCAGCTCGGTGCACCGCCACCCCTTCCTCAATC
TGAACACCATGCGCCCGCGGCTGCGCTACAGCCCCTACTCCATCCCGGTGCCGGTCCCG
GACGGCAGCAGTCTGCTCACCACCGCCCTGCCCTCCATGGCGGCGGCCGCGGGGCCCCT
GGACGGCAAAGTCGCCGCCCTGGCCGCCAGCCCGGCCTCGGTGGCAGTGGACTCGGGCT
CTGAACTCAACAGCCGCTCCTCCACGCTCTCCTCCAGCTCCATGTCCTTGTCGCCCAAA
CTCTGCGCGGAGAAAGAGGCGGCCACCAGCGAACTGCAGAGCATCCAGCGGTTGGTTAG
CGGCTTGGAAGCCAAGCCGGACAGGTCCCGCAGCGCGTCCCCG

[0061] In certain embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 2-30 contiguous in length and is at least about 80% homologous (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to at least about 2 to about 30 contiguous nucleotides of a human TBX3 cDNA sequence. In some embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 2-30 contiguous in length and is at least about 80% homologous (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to at least about 2 to about 30 contiguous nucleotides of the human TBX3 sequence RefSeq: NM_005996.4 or a variant thereof. In some embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 2-30 contiguous in length and is at least about 80% homologous (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to at least about 2 to about 30 contiguous nucleotides of SEQ ID NO: 1. In some other embodiments, siRNA molecules disclosed herein may have a nucleotide sequence that is about 2-30 contiguous in length and is homologous to at least about 2 to about 30 contiguous nucleotides of SEQ ID NO: 1.

[0062] A siRNA molecule disclosed herein may be synthesized in any number of conformations. One skilled in the art would recognize the type of siRNA conformation to be used for a particular purpose. Examples of siRNA conformations include, but need not be limited to, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single-stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having complementary sense and antisense regions; or a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions. In the case of the circular polynucleotide, the polynucleotide may be processed either in vivo or in vitro to generate an active double-stranded siRNA molecule.

[0063] In certain embodiments, siRNA molecules disclosed herein may be double stranded siRNA molecules. In some aspects, double stranded siRNA molecules disclosed herein may have at least one sense sequence. In some other aspects, double stranded siRNA molecules disclosed herein may have at least one antisense sequence. In still some other aspects, double stranded siRNA molecules disclosed herein may have at least one antisense sequence and at least one sense sequence. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one antisense sequence selected from Table 1, at least one sense sequence selected from Table 1, or both.

TABLE-US-00002 TABLE 1 Sense Sequence Over- Antisense Sequence Over- siRNA ID (5′-3′) hang (5′-3′) hang TBX3 470 GCUGAUGACUGUCGUUAUA UU UAUAACGACAGUCAUCAGC UU (SEQ ID NO: 2) (SEQ ID NO: 59) TBX3 1080 GGACAUCGAACCUCAAAGA UU UCUUUGAGGUUCGAUGUCC UU (SEQ ID NO: 3) (SEQ ID NO: 60) TBX3 457 GGACAUUAUAGCUGCUGAU UU AUCAGCAGCUAUAAUGUCC UU (SEQ ID NO: 4) (SEQ ID NO: 61) TBX3 707 GGUACUCAGACUAUAUUGA UU UCAAUAUAGUCUGAGUACC UU (SEQ ID NO: 5) (SEQ ID NO: 62) TBX3 754 CCACAUUGUAAGAGCCAAU UU AUUGGCUCUUACAAUGUGG UU (SEQ ID NO: 6) (SEQ ID NO: 63) TBX3 966 GGGUGUUUGAUGAAAGACA UU UGUCUUUCAUCAAACACCC UU (SEQ ID NO: 7) (SEQ ID NO: 64) TBX3 631 CAACAUUUCAGACAAACAU UU AUGUUUGUCUGAAAUGUUG UU (SEQ ID NO: 8) (SEQ ID NO: 65) TBX3 628 CAACAACAUUUCAGACAAA UU UUUGUCUGAAAUGUUGUUG UU (SEQ ID NO: 9) (SEQ ID NO: 66) TBX3 627 CCAACAACAUUUCAGACAA UU UUGUCUGAAAUGUUGUUGG UU (SEQ ID NO: 10) (SEQ ID NO: 67) TBX3 857 GAUAAGAUAACCCAGUUAA UU UUAACUGGGUUAUCUUAUC UU (SEQ ID NO: 11) (SEQ ID NO: 68) TBX3 389- CGAAUGUUUCCUCCAUUUA AA UAAAUGGAGGAAACAUUCG CC 411 (SEQ ID NO: 12) (SEQ ID NO: 69) TBX3 390- GAAUGUUUCCUCCAUUUAA AG UUAAAUGGAGGAAACAUUC UU 412 (SEQ ID NO: 13) (SEQ ID NO: 70) TBX3 438- CCAAAUACAUUUUAUUGAU UU AUCAAUAAAAUGUAUUUGG CU 460 (SEQ ID NO: 14) (SEQ ID NO: 71) TBX3 619- GAAACUCACCAACAACAUU UC AAUGUUGUUGGUGAGUUUC AG 641 (SEQ ID NO: 15) (SEQ ID NO: 72) TBX3 768- CCAAUGACAUCUUGAAACU CC AGUUUCAAGAUGUCAUUGG CU 790 (SEQ ID NO: 16) (SEQ ID NO: 73) TBX3 917- GGAAAUGGCCGAAGAGAAA AA UUUCUCUUCGGCCAUUUCC AG 939 (SEQ ID NO: 17) (SEQ ID NO: 74) TBX3 428- GAUAAAAAAGCCAAAUACA UU UGUAUUUGGCUUUUUUAUC CA 450 (SEQ ID NO: 18) (SEQ ID NO: 75) TBX3 437- GCCAAAUACAUUUUAUUGA UG UCAAUAAAAUGUAUUUGGC UU 459 (SEQ ID NO: 19) (SEQ ID NO: 76) TBX3 778- CUUGAAACUCCCUUAUAGU AC ACUAUAAGGGAGUUUCAAG AU 800 (SEQ ID NO: 20) (SEQ ID NO: 77) TBX3 860- AAGAUAACCCAGUUAAAAA UU UUUUUAACUGGGUUAUCUU AU 882 (SEQ

ID NO: 21) (SEQ ID NO: 78) J-012197- CAUCGAACCUCAAAGAUUU UU AAAUCUUUGAGGUUCGAUG UU 05 (SEQ ID NO: 22) (SEQ ID NO: 79) J-012197- CCAAAGAGGAUGUACAUUC UU GAAUGUACAUCCUCUUUGG UU 06 (SEQ ID NO: 23) (SEQ ID NO: 80) J-012197- CAUUAUAGCUGCUGAUGAC UU GUCAUCAGCAGCUAUAAUG UU 07 (SEQ ID NO: 24) (SEQ ID NO: 81) J-012197- UAUAGUACAUUUCGGACAU UU AUGUCCGAAAUGUACUAUA UU 08 (SEQ ID NO: 25) (SEQ ID NO: 82) D-012197- CAUCGAACCUCAAAGAUUU UU AAAUCUUUGAGGUUCGAUG UU 01 (SEQ ID NO: 26) (SEQ ID NO: 83) D-012197- GCUGAUGACUGUCGUUAUA UU UAUAACGACAGUCAUCAGC UU 02 (SEQ ID NO: 27) (SEQ ID NO: 84) D-012197- CCAAUGACAUCUUGAAACU UU AGUUUCAAGAUGUCAUUGG UU 03 (SEQ ID NO: 28) (SEQ ID NO: 85) D-012197- CCAUUUAAAGUGAGAUGUU UU AACAUCUCACUUUAAAUGG UU 04 (SEQ ID NO: 29) (SEQ ID NO: 86) s13865 GAAACUCCCUUAUAGUACA TT UGUACUAUAAGGGAGUUUC AA (SEQ ID NO: 30) (SEQ ID NO: 87) s13866 CCAACAACAUUUCAGACAA TT UUGUCUGAAAUGUUGUUGG TG (SEQ ID NO: 31) (SEQ ID NO: 88) s13867 GCUGAUGACUGUCGUUAUA TT UAUAACGACAGUCAUCAGC AG (SEQ ID NO: 32) (SEQ ID NO: 89) s529425 AAGUGAGACUAUUAGACAA TT UUGUCUAAUAGUCUCACUU CT (SEQ ID NO: 33) (SEQ ID NO: 90) s534311 CCAUUUAAAGUGAGAUGUU TT AACAUCUCACUUUAAAUGG AG (SEQ ID NO: 34) (SEQ ID NO: 91) s534312 GAUAACCCAGUUAAAAAUA TT UAUUUUUAACUGGGUUAUC TT (SEQ ID NO: 35) (SEQ ID NO: 92) 107921 GGGUGUUUGAUGAAAGACA TT UGUCUUUCAUCAAACACCC TC (SEQ ID NO: 36) (SEQ ID NO: 93) 107922 GGCUCACCUUUUCGCUGCU TT AGCAGCGAAAAGGUGAGCC TT (SEQ ID NO: 37) (SEQ ID NO: 94) 115750 GCAGCUUUCAACUGCUUCG TT CGAAGCAGUUGAAAGCUGC TT (SEQ ID NO: 38) (SEQ ID NO: 95) 115751 CCUCAAAGAUUUAUGUCCC TT GGGACAUAAAUCUUUGAGG TT (SEQ ID NO: 39) (SEQ ID NO: 96) 139810 GGAGUGGGCAAAACACAUA TT UAUGUGUUUUGCCCACUCC TT (SEQ ID NO: 40) (SEQ ID NO: 97) 239194 UGGCCGAAGAGAAAAAAGA TT UCUUUUUUCUCUUCGGCCA TT (SEQ ID NO: 41) (SEQ ID NO: 98) 239195 CAAGCAGCUUUCAACUGCU TT AGCAGUUGAAAGCUGCUUG TT (SEQ ID NO: 42) (SEQ ID NO: 99) 239196 AACUCCCUUAUAGUACAUU TT AAUGUACUAUAAGGGAGUU TC (SEQ ID NO: 43) (SEQ ID NO: 100) 3661 GGCCUUUGAAGACCAUGGA TT UCCAUGGUCUUCAAAGGCC TC (SEQ ID NO: 44) (SEQ ID NO: 101) 3754 GGUGCACCUGGAGGCUAAA TT UUUAGCCUCCAGGUGCACC TT (SEQ ID NO: 45) (SEQ ID NO: 102) 3845 GGCUAAAGAACUUUGGGAU TT AUCCCAAAGUUCUUUAGCC TC (SEQ ID NO: 46) (SEQ ID NO: 103) TBX3 186 CGAUCAUGGAUCAAUUGGU UU ACCAAUUGAUCCAUGAUCG UU (SEQ ID NO: 47) (SEQ ID NO: 104) TBX3 454 GAUGGACAUUAUAGCUGCU UU AGCAGCUAUAAUGUCCAUC UU (SEQ ID NO: 48) (SEQ ID NO: 105) TBX3 589 GAUGUCCAAAGUCGUCACU UU AGUGACGACUUUGGACAUC UU (SEQ ID NO: 49) (SEQ ID NO: 106) TBX3 540 CAAAGAGGAUGUACAUUCA UU UGAAUGUACAUCCUCUUUG UU (SEQ ID NO: 50) (SEQ ID NO: 107) TBX3 781 GAAACUCCCUUAUAGUACA UU UGUACUAUAAGGGAGUUUC UU (SEQ ID NO: 51) (SEQ ID NO: 108) TBX3 713 CAGACUAUAUUGAACUCCA UU UGGAGUUCAAUAUAGUCUG UU (SEQ ID NO: 52) (SEQ ID NO: 109) TBX3 368 GUCAUUACCAAGUCGGGAA UU UUCCCGACUUGGUAAUGAC UU (SEQ ID NO: 53) (SEQ ID NO: 110) TBX3 324 CUAAAGAACUUUGGGAUCA UU UGAUCCCAAAGUUCUUUAG UU (SEQ ID NO: 54) (SEQ ID NO: 111) TBX3

788 CCUUAUAGUACAUUUCGGA UU UCCGAAAUGUACUAUAAGG UU (SEQ ID NO: 55) (SEQ ID NO: 112) TBX3 717 CUAUAUUGAACUCCAUGCA UU UGCAUGGAGUUCAAUAUAG UU (SEQ ID NO: 56) (SEQ ID NO: 113) TBX3 820 CGAAACUGAAUUCAUCGCU UU AGCGAUGAAUUCAGUUUCG UU (SEQ ID NO: 57) (SEQ ID NO: 114) TBX3 1091 CUCAAAGAUUUAUGUCCCA UU UGGGACAUAAAUCUUUGAG UU (SEQ ID NO: 58) (SEQ ID NO: 115)

[0064] In some embodiments, double stranded siRNA molecules disclosed herein may have at least one sense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 2-58. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one antisense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 59-115. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one sense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 2-58 and at least one antisense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 59-115. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one sense sequence of SEQ ID NOs: 2-58. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one antisense sequence of SEQ ID NOs: 59-115. In some embodiments, double stranded siRNA molecules disclosed herein may have at least one sense sequence of SEQ ID NOs: 2-58 and at least one antisense sequence of SEQ ID NOs: 59-115. In some aspects, double stranded siRNA molecules disclosed herein may have at least one sense sequence of SEQ ID NOs: 2-21 and at least one antisense sequence of SEQ ID NOs: 59-78. In some other aspects, double stranded siRNA molecules disclosed herein may have at least one sense sequence of SEQ ID NOs: 47-58 and at least one antisense sequence of SEQ ID NOs: 104-155. In some aspects, double stranded siRNA molecules disclosed herein may have a sense sequence of SEQ ID NO: 3 and an antisense sequence of SEQ ID NO: 60. In some aspects, double stranded siRNA molecules disclosed herein may have a sense sequence of SEQ ID NO: 18 and an antisense sequence of SEQ ID NO: 75. In some aspects, double stranded siRNA molecules disclosed herein may have a sense sequence of SEQ ID NO: 19 and an antisense sequence of SEQ ID NO: 76.

[0065] In some embodiments, the present disclosure also encompasses nucleic acid sequences encoding a double stranded siRNA molecule as disclosed herein. In some embodiments, a nucleic acid sequence encoding the double stranded siRNA as disclosed herein may be a double stranded DNA, single stranded DNA, a plasmid vector, a viral vector for example a retroviral vector, lentiviral vector, a pox viral vector, an adenoviral vector, or an adeno-associated viral vector. In some embodiments, the nucleic acid may encode at least one sense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 2-58. In some embodiments, the nucleic acids may encode at least one antisense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 59-115. In some embodiments, the nucleic acids may encode at least one sense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 2-58 and at least one antisense sequence having at least about 80% homology (e.g., about 80%, about 85%, about 90%, about 95%, or about 99%) to any one of SEQ ID NOs: 59-115. In some embodiments, the nucleic acids may encode at least one sense sequence of any one of SEQ ID NOs: 2-58. In some embodiments, the nucleic acids may encode at least one antisense sequence of any one of SEQ ID NOs: 59-115. In some embodiments, the nucleic acids may encode at least one sense sequence of SEQ ID NOs: 2-58 and at least one antisense sequence of SEQ ID NOs: 59-115. In some embodiments, the nucleic acid may encode a sense sequence of SEQ ID NO: 3 and an antisense sequence of SEQ ID NO: 60. In some aspects, the nucleic acid may encode a sense sequence of

SEQ ID NO: 18 and an antisense sequence of SEQ ID NO: 75. In some aspects, nucleic acid molecule may encode a sense sequence of SEQ ID NO: 19 and an antisense sequence of SEQ ID NO: 76.

[0066] The present disclosure also provides for methods of making the siRNA molecules disclosed herein. Making of siRNA molecules disclosed herein may be through chemical synthesis or siRNA molecules disclosed herein may be encoded by a plasmid and transcribed or may be vectored by a virus engineered to express the siRNA. A siRNA molecule disclosed herein may be a single stranded molecule with complementary sequences that self-hybridize into duplexes with hairpin loops. An siRNA molecule disclosed herein can also be generated by cleavage of parent dsRNA through the use of an appropriate enzyme such as *E. coli* RNase III or Dicer. A parent dsRNA may be any double stranded RNA duplex from which a siRNA may be produced, such as a full or partial mRNA transcript. Use of cell lysates or in vitro processing may further involve the subsequent isolation of the short, nucleotide siRNAs (e.g., about 2-25 nucleotides in length) from the lysate, etc., making the process somewhat cumbersome and expensive. Chemical synthesis proceeds by making two single stranded RNA-oligomers followed by the annealing of the two single stranded oligomers into a double stranded RNA. Methods of chemical synthesis are diverse and can be easily adapted to synthesis different siRNAs.

[0067] In certain embodiments, siRNA molecules disclosed herein may abolish gene expression of TBX3. Methods known in the art for the detection and quantification of RNA expression suitable for use herein can include, but are not limited to northern blotting and in situ hybridization, RNAse protection assays, polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), real-time quantitative reverse transcription PCR (RT-qPCR or qPCR), sequencing-based gene expression analysis (e.g., Serial Analysis of Gene Expression (SAGE)), gene expression analysis by massively parallel signature sequencing (MPSS), and the like.

[0068] The siRNA molecules of the present disclosure specifically downregulate gene expression of TBX3 (T-Box Transcription Factor 3) or a variant thereof. The term downregulating may be interchangeably expressed as reducing, inhibiting, preventing, blocking or silencing. Here, the phrase “downregulating gene expression” refers to any reduced level of gene expression in comparing with an ordinary expression level. For example, the reduced level of gene expression can be from about 70% to about 0% of the ordinary expression level. In other words, about 30% to about 100% gene expression is downregulated, reduced, blocked, inhibited, prevented or silenced, comparing to the ordinary expression level. Specifically, the reduced level of gene expression is about 70%, 65%, 60%, 55%, 50%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 8%, 5%, 4%, 3%, 2%, 1% or 0% of the ordinary expression level of TBX3. The reduced level of gene expression can also be any percentage or range as recited above. On the other hand, the term “specific” or “specifically” used in combination with downregulating refers to downregulation of a target gene's expression with minimal or no binding or downregulation of other nucleic acids or their expressions.

[0069] In some embodiments, siRNA molecules disclosed herein may reduce gene expression of TBX3 by at least about 50%. In some aspects, siRNA molecules disclosed herein may reduce gene expression of TBX3 by about 50% to about 99%, about 55% to about 98%, or about 60% to about 95%. In some aspects, siRNA molecules disclosed herein may reduce gene expression of TBX3 by about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99%.

[0070] In certain embodiments, siRNA molecules disclosed herein may abolish protein expression of TBX3. Methods known in the art for the detection and quantification of protein expression suitable for use herein can include, but are not limited to ELISAs (enzyme-linked immunosorbent assays), immunoblot assays, flow cytometric assays, immunohistochemical assays, radioimmuno assays, Western blot assays, an immunofluorescent assays, chemiluminescent assays, mass spectrometry assays, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass

mapping, liquid chromatography/quadrupole time-of-flight electrospray ionization tandem mass spectrometry (LC/Q-TOF-ESI-MS/MS), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and the like.

[0071] In some embodiments, siRNA molecules disclosed herein may reduce protein expression of TBX3 by at least about 50%. In some aspects, siRNA molecules disclosed herein may reduce protein of TBX3 by about 50% to about 99%, about 55% to about 98%, or about 60% to about 95%. In some other aspects, siRNA molecules disclosed herein may reduce protein expression of TBX3 by about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99%.

[0072] In some embodiments, siRNA molecules disclosed herein may have one or more chemical modifications. Non-limiting examples of chemical modifications can include terminal cap moieties, phosphate backbone modifications, and the like. Examples of classes of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4′,5′-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4′-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, threo pentofuranosyl nucleotides, acyclic 3′,4′-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3′-3′-inverted nucleotide moieties, 3′-3′-inverted abasic moieties, 3′-2′-inverted nucleotide moieties, 3′-2′-inverted abasic moieties, 5′-5′-inverted nucleotide moieties, 5′-5′-inverted abasic moieties, 3′-5′-inverted deoxy abasic moieties, 5′-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3 aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3′-phosphoramidate, 5′ phosphoramidate, hexylphosphate, aminohexyl phosphate, 3′-phosphate, 5′-amino, 3′-phosphorothioate, 5′-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5′-mercapto moieties. Non-limiting examples of phosphate backbone modifications (i.e., resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions. Such chemical modifications can occur at the 5′-end and/or 3′-end of the sense strand, antisense strand, or both strands of a siRNA molecule disclosed herein.

[0073] Chemical modification of a siRNA molecule disclosed herein may comprise modification of at least one ribosugar moiety of its nucleotide sequence. The ribosugar moiety may be modified with 2 2′-O-methyl (2′OMe), 2′-deoxy-2′-fluoro (2′F), 2′-deoxy, 5-C-methyl, 2′-O-(2-methoxyethyl) (MOE), 4′-thio, 2′-amino, 2′-C-allyl, or any combination thereof. In some aspects, a siRNA molecule disclosed herein may have less than about 10% to about 70% (e.g., about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%) of ribosugar moieties of the total nucleotide sequence modified.

[0074] Chemical modification of a siRNA molecule disclosed herein may comprise attaching a conjugate to the siRNA molecule. The type of conjugate used and the extent of conjugation to the siRNA can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify siRNA conjugates having improved properties using any of a variety of well-known in vitro cell culture or in vivo animal models including the negative-controlled expression studies described above. The conjugate can be attached at the 5′- and/or the 3′-end of the sense and/or the antisense strand of the siRNA via a covalent attachment such as a nucleic acid or non-nucleic acid linker. The conjugate can be attached to the siRNA through a carbamate group or other linking group (see, e.g., U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727, the content of each of which is incorporated by reference herein in its entirety). A conjugate may be added to siRNA for any of a number of purposes. For example, the conjugate may be a molecular entity that facilitates the

delivery of siRNA into a cell or may be a molecule that comprises a drug or label. Examples of conjugate molecules suitable for attachment to siRNA of the present invention include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (e.g., folic acid, folate analogs and derivatives thereof), sugars (e.g., galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, etc.), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof. Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in e.g., U.S. Patent Publication Nos. 20050119470 and 20050107325, the content of each of which is incorporated by reference herein in its entirety. Other examples include the 2′-O-alkyl amine, 2′-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules, and the like. Additional examples of conjugate molecules include a hydrophobic group, a membrane active compound, a cell penetrating compound, a cell targeting signal, an interaction modifier, or a steric stabilizer as described in U.S. Patent Publication No. 20040167090, incorporated by reference herein in its entirety.

[0075] In certain embodiments, siRNA molecules disclosed herein may be conjugated to at least one targeting ligand. Targeting ligands contemplated herein include ligands suitable for targeting siRNA molecules to a liver, a liver tissue, and/or a liver cell. Non-limiting examples of targeting ligands suitable for use herein may include galactose, galactosamine, N-formyl-galactosamine, N-acetylgalactosamine, N-propionyl-galactosamine, N-n-butanoyl-galactosamine, N-iso-butanoylgalactos-amine, galactose cluster, and N-acetylgalactosamine trimer and may optionally have a pharmacokinetic modulator selected from the group consisting of: hydrophobic group having 16 or more carbon atoms, hydrophobic group having 16-20 carbon atoms, palmitoyl, hexadec-8-enoyl, oleyl, (9E,12E)-octadeca-9,12dienoyl, dioctanoyl, and C16-C20 acyl, and cholesterol. In some embodiments, a liver targeting ligand suitable for use herein may be a N-Acetylgalactosamine (GalNAc) conjugate. In some embodiments, siRNA molecules disclosed herein may be conjugated to at least one GalNAc conjugate. In some embodiments, siRNA molecules disclosed herein may be conjugated to about 1 to about 10 GalNAc conjugates, about 2 to about 9 GalNAc conjugates, or about 3 to about 8 GalNAc conjugates. In some embodiments, siRNA molecules disclosed herein may be conjugated to about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 GalNAc conjugates.

[0076] Any of the siRNA molecules disclosed herein may target the open reading frame or the 5′ or 3′ UTRs of the TBX3 gene or at least one variant thereof.

[0077] siRNA molecules disclosed herein can specifically downregulate gene expression of at least one variant of TBX3. The TBX3 gene and/or its variant may be associated with a liver disease, liver disfunction, liver injury, and/or liver damage. The liver disease may comprise fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer, or any combination thereof. Liver damage may include cirrhosis, chronic infection of hepatitis B virus (HBV), chronic infection of hepatitis C virus (HCV), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis (PBC), hereditary hemochromatosis, type 2 diabetes, obesity, tobacco use, alcohol abuse, long-term anabolic steroid use, tyrosinemia, alpha1-antitrypsin deficiency, porphyria cutanea tarda, glycogen storage diseases, Wilson disease, or any combination thereof. In some embodiments, a suitable subject for the methods herein may have or be suspected of having one or more injuries to the liver that may predispose a subject to HCC.

[0078] In certain embodiments, methods and compositions provided herein can include a vector containing any one of the siRNA molecules disclosed herein. In some embodiments, a vector for

use herein can be a viral vector. As used herein, the term “viral vector” can refer to a nucleic acid vector construct that includes at least one element of viral origin and has the capacity to be packaged into a viral vector particle and encodes at least an exogenous polynucleotide. In certain embodiments, the vector and/or particle can be utilized for the purpose of transferring any nucleic acids into cells either in vitro or in vivo. Numerous viral vectors are known in the art. The term virion can refer to a single infective viral particle. “Viral vector”, “viral vector particle” and “viral particle” also refer to a complete virus particle with its DNA or RNA core and protein coat as it exists outside the cell. Non-limiting examples of viral vectors for use herein can include adenoviruses, adeno-associated viruses (AAV), herpesviruses, retroviruses, lentiviruses, integrase defective lentiviruses (IDLV), and the like. In some embodiments, a viral vector disclosed herein can be a lentiviral vector. Examples of lentiviruses include, but are not limited to, human lentiviruses such as HIV (in particular HIV-1 or HIV-2), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), Caprine Arthritis Encephalitis Virus (CAEV), visna and progressive pneumonia viruses of sheep, baboon pseudotype viruses, bovine immunodeficiency virus (BIV), and the like. In some embodiments, siRNA molecules and/or vectors described herein can be prepared by conventional recombinant technology known to one of skill in the art. In other embodiments, siRNA molecules and/or vectors described herein can be prepared by a gene editing method known in the art (e.g., by CRISPR). In certain embodiments, methods provided herein can include generating a cell to express any of the siRNA molecules and/or vectors described herein. In some embodiments, vectors, viral particles, and the like as contemplated herein may be encapsulated into a liposome for delivery to a subject.

[0079] In some aspects, the current disclosure also encompasses use of gene editing systems for example CRISPR based systems for abolishing or downregulating gene expression of TBX3. As such, the current disclosure also encompasses compositions comprising a nucleic acid sequence related to a cluster regularly interspaced short palindromic repeats (CRISPR) system, for example a single guide RNA (sgRNA), a CRISPR-RNA (crRNA), a trans-activating crRNA (tracrRNA), and further a plasmid DNA (pDNA) or a viral vector encoding nucleic acid sequence related to a cluster regularly interspaced short palindromic repeats (CRISPR) system, that specifically target TBX3 or variants thereof. In some aspects, the gene editing system comprises at least an sgRNA targeting TBX3 or a UTR thereof and an RNA guided endonuclease for example Cas9. In some aspects, the gene editing system comprises at least an sgRNA targeting TBX3 and an RNA guided endonuclease for example Cas9. In some aspects, the sgRNA target sequence is at least about 80% identical to any one of SEQ ID NO: 118-217 as provided in Table 2. In some aspects, the sgRNA targets a sequence at least about 80%, or about 85%, or about 90%, or about 95% or about 100% identical to SEQ ID NO: 118-217. In some aspects, the sgRNA targets 20 consecutive nucleotides in any one of SEQ ID NO: 218-317. None of the gRNA as provided in Table 2 are predicted to target the last exon or have 10 bp off target match.

TABLE-US-00003 TABLE 2 Guide with flanking sequence (10 bp cut SEQ before and SEQ position On- Off- gRNA ID after the ID in target target Sequence NOS guide + PAM) NOS chromosome efficiency Exon score GAAAAGGTGA 118 GCTCAGCAGC 218 115112508 0.714618143 7 0 GCCTTGACCG GAAAAGGTGA GCCTTGACCG CGGCGGGGC TGCC GCCCCGCTAC 119 CACCCGGACA 219 115118882 0.711616088 2 0.353571 TGGGGAACA GCCCCGCTAC G TGGGGAACA GTGGTGGATG TCCA CAAGATCTCC 120 GCGACGCGG 220 115112068 0.708326443 7 0 ACCACCACGT CCAAGATCTC CACCACCACG TCGGCGGAG GAGCC CGAGGGTGA 121 TATGTCCCAG 221 115112002 0.692170265 7 0.059091 GAGCGACGC CGAGGGTGA CG GAGCGACGC CGAGGAGGC CGAGAG TGTCATTGGC 122 AGTTTCAAGA 222 115117415 0.684737218 4 0 TCTTACAATG TGTCATTGGC TCTTACAATG TGGTGGAACC GGG AAAGAGGATG 123 CCGAAATGCC 223 115118856 0.677873292 2 0 TACATTCACC AAAGAGGATG TACATTCACC CGGCGGACA GCCC GTTCGATGTC 124 TACCTTTGAG 224 115114144 0.674367102 6 0

CCTACAGTGG GTTCGATGTC CCTACAGTGG AGGAGGCGG CTGG GAACATCTCA 125
ATCCAGCCCA 225 115118938 0.672438822 2 0.109804 CTTTAAATGG GAACATCTCA
CTTTAAATGG AGGAGGAAAC ATT CCGACCCCG 126 GCTGGTAAGG 226 115118840
0.671949373 2 0 AAATGCCAAA CCGACCCCG G AAATGCCAAA GAGGAGGAT GTACA
CAAAGAGGA 127 AGGCCGAGA 227 115112032 0.669845158 7 0.043831 GCATGGCCC
GCAAAGAGGA CG GCATGGCCC CGAGGAGGC CTGCGA ATCCAGGCCC 128
TGGCCGTGG 228 115112088 0.658171921 7 0 GAGACACCG AATCCAGGCC G
CGAGACACC GGTGGTGGA GGCCCC TTCACTGGAG 129 AAGCTGCTTG 229 115114210
0.653146782 6 0 GACTCATCAG TTCACTGGAG GACTCATCAG AGGAGGTCC CATT
GAAAGCTGCT 130 CGAAGCAGTT 230 115114198 0.6492486 6 0 TGTTCACTGG
GAAAGCTGCT TGTTCACTGG AGGAGGACTC ATC GAGGTTCGAT 131 GAGGTTCGAT
231 115114141 0.64192681 6 0 GTCCCTACAG GTCCCTACAG TGGTGGAGG CGGC
AGCTCACCCT 132 CACAGAAAAC 232 115114142 0.624268231 6 0.00293 GCAGTCCATG
AGCTCACCCT GCAGTCCATG AGGAGGGTG TTTG GCCGAGAGC 133 CGACGCCGA 233
115112024 0.619070831 7 0 AAAGAGGAG GGCCGAGAG CA CAAAGAGGAG
CATGGTGGCC CCGAG CATCCACTGT 134 CGACTTTGGA 234 115118766 0.618411118 2 0
TCCCCAGTAG CATCCACTGT TCCCCAGTAG CGGCGGGGC TGTC CCATGCACAA 135
ATATTGAACT 235 115117342 0.617360315 4 0.046524 ATACCAGCCC CCATGCACAA
ATACCAGCCC CGGCGGTTC CACA ATAAATTTCA 136 GACTGTCGTT 236 115118801
0.598188657 2 0 CAATTCTCGG ATAAATTTCA CAATTCTCGG TGGTGGATGG TGG
GCGTCCGTCT 137 CGCGGCGGC 237 115112296 0.5963183 7 0 GCACCGTGA
CGCGTCCGTC G TGCACCGTGA GCGGCGGCG CGAAG TGTCTCGGGC 138
CCTCCACCGG 238 115112545 0.594369303 7 0 CTGGATTCCA TGTCTCGGGC
CTGGATTCCA CGGCGGCCA TGGC GGTGCCCGTT 139 AGGAAGAGC 239 115112210
0.593081981 7 0 GAAGAACTGT GGGTGCCCG TTGAAGAACT GTTGGTGGCC CGCCA
TCACAATTCT 140 GTTATAAATTT 240 115118808 0.591282693 2 0 CGGTGGATG
CACAATTCTC G GGTGGATGGT GGTGGCTGG TAA CGATGTCCCT 141 CTTTGAGGTT
241 115114147 0.589956292 6 0 ACAGTGGAG CGATGTCCCT G ACAGTGGAG
GCGGCGGCT GGAGA CCCCAGAAAC 142 CCGGTGGAG 242 115112114 0.587176141 7 0
CGTGGCCAG GCCCCAGAAA G CCGTGGCCA GGAGGAGGG GACCCA TTTGTGGAAA
143 TGAGTTTCAG 243 115118742 0.576703566 2 0 GTGACGACTT TTTGTGGAAA
GTGACGACTT TGGTGGACAT CCA CAGGCCCGA 144 CCGTGGAATC 244 115112091
0.564917969 7 0.037427 GACACCGGT CAGGCCCGA GG GACACCGGT GGAGGAGGC
CCCAGA GGACAGTCCC 145 CGGACGCCC 245 115112046 0.562625974 7 0.007792
TGCGCCGCA CGGACAGTCC G CTGCGCCGC AGCGGCGGC AGAGGC TGGCCCCGA 146
AAGAGGAGCA 246 115112044 0.562084199 7 0 GGCCTGCGA TGGCCCCGA CG
GGCCTGCGA CGCGGCGGC CAAGAT TCTTTCATCA 147 CCTTTTTGTG 247 115114252
0.547366511 6 0 AACACCCTCA TCTTTCATCA AACACCCTCA TGGTGGACTG CAG
GGAGGAGCC 148 CCACCACGTC 248 115112089 0.545951103 7 0 CTGCCGTGAC
GGAGGAGCC A CTGCCGTGAC AAGGAGGGC AGCCC GGGGCCGCT 149 CCGCTGTCCC
249 115112492 0.542944147 7 0 CAGCAGCGAA GGGGCCGCT A CAGCAGCGAA
AAGGAGGTGA GCCT GCGCAGGAG 150 GCGCGGAGG 250 115112248 0.542164198 7 0
CCCGGTTCGC AGCGCAGGA G GCCCGGTTC GCGAGGAGG GCACAGC GGTTTCCGGG
151 TTTTGCAAAA 251 115115443 0.541096538 5 0.046753 ACACTGGAAA GGTTTCCGGG
ACACTGGAAA TGGTGGCCG AAGA AGGGAGTTTC 152 ATGTACTATA 252 115117401
0.540968892 4 0 AAGATGTCAT AGGGAGTTTC AAGATGTCAT TGGTGGCTCT TAC
CCGGGGCTG 153 CAATGTGGAA 253 115117440 0.539444587 4 2.87266 GTATTTGTGC
CCGGGGCTG A GTATTTGTGC ATGGTGGAGT TCAA ATTTCACAATT 154 GTCGTTATAA
254 115118805 0.530626362 2 0 CTCGGTGGA ATTTCACAATT CTCGGTGGAT
GGTGGTGGC TGG CACTCGCGG 155 TCTCGTCCAG 255 115112224 0.527300954 7 0

CCTGGGCGC CACTCGCGG GG CCTGGGCGC GGAGGAGGA GCGCAG AGGCCCCAG
156 ACACCGGTG 256 115112111 0.526292158 7 0 AAACCGTGGC GAGGCCCCA C
GAAACCGTGG CCAGGAGGA GGGGAC CCGCGGGGC 157 TGAGCCTTGA 257 115112525
0.526023177 7 0.059524 TGCCCTTGTC CCGCGGGGC A TGCCCTTGTC ACGGCGGCA
GGGCT GCGGGCCAA 158 CCCGGGCCT 258 115112411 0.517318009 7 0 CAGTTCTTCA
GGCGGGCCA A ACAGTTCTTC AACGGCGGG CACCCG TCTCCAGCCG 159
CCAGGCTTCT 259 115114255 0.513501316 6 0.02795 CCTCCACTGT TCTCCAGCCG
CCTCCACTGT AGGAGGGAC ATCG TAAAGTGAGA 160 TTCCTCCATT 260 115118
0.51348377 2 0.023228 TGTTCTGGGC TAAAGTGAGA 721 TGTTCTGGGC TGGTGGATAA
AAA AGCTTTCAAC 161 GTGAACAAGC 261 115114227 0.512276609 6 0 TGCTTCGCCC
AGCTTTCAAC TGCTTCGCCC AGGAGGCTTC TTC TTCGGCCATT 162 CTTTTTTCTCT
262 115115414 0.511671134 5 0 TCCAGTGTCC TCGGCCATTT CCAGTGTCCC
GGCGGAAAC CTT GTTGAAAGCT 163 GGGCGAAGC 263 115114195 0.511455039 6
0.012245 GCTTGTTCAC AGTTGAAAGC TGCTTGTTCA CTGGTGGAG GACTC
GCCATGCTCC 164 AGGCCTCGG 264 115112601 0.51141366 7 0 TCTTTGCTCT
GGCCATGCTC CTCTTTGCTC TCGGCGGCC TCGGC AATTCTCGGT 165 TAAATTTCAC
265 115118812 0.502328709 2 0.131868 GGATGGTGG AATTCTCGGT C GGATGGTGG
CTGGTGGTAA GGCC CGCGCCGCT 166 AGGAGGCCTT 266 115112326 0.500475435 7 0
CACGGTGCA CGCGCCGCT GA CACGGTGCA GACGGCGGA CGCGGC GGGACTGTCC
167 CTGCGGCGC 267 115112593 0.498017929 7 0.018803 GGGGCGTCC AGGGACTGTC G
CGGGGCGTC CGCGGCGGC CACCCT GCAAAAGGTT 168 CAACCCTTTT 268 115115437
0.4925993 5 0.668662 TCCGGGACAC GCAAAAGGTT TCCGGGACAC TGGTGGAAAT GGC
ATGTTTGTCT 169 TTACAAATCC 269 115118709 0.492461709 2 0 GAAATGTTGT
ATGTTTGTCT GAAATGTTGT TGGTGGTGAG TTT TTACAATGTG 170 TCATTGGCTC 270
115117427 0.491623031 4 0 GAACCGGGG TTACAATGTG C GAACCGGGG CTGGTGGTAT
TTGT CAAGGGCAG 171 CCTGCCGTGA 271 115112107 0.491507388 7 0 CCCCGCGGT
CAAGGGCAG CA CCCCGCGGT CAAGGAGGCT CACCT CAAACACCCT 172
TGTCTTTCAT 272 115114260 0.483843367 6 0 CATGGACTGC CAAACACCCT
CATGGACTGC AGGAGGGTG AGCT GTCCCTACAG 173 GAGGTTCGAT 273 115114151
0.479159771 6 0 TGGAGGCGG GTCCCTACAG C TGGAGGCGG CTGGTGGAGA AGAA
CCTCTTTGGC 174 GAATGTACAT 274 115118811 0.476893705 2 0 ATTTCGGGGT
CCTCTTTGGC ATTTCGGGGT CGGCGGCCT TACC CAGCACTCGC 175 CCATCTCGTC 275
115112221 0.469561023 7 0 GGCCTGGGC CAGCACTCGC G GGCCTGGGC GCGGCGGAG
GAGCG AACAACATTT 176 GAAACTCACC 276 115118944 0.46931656 2 0.123077
CAGACAAACA AACAACATTT CAGACAAACA TGGTGGATTT GTA GTTTGATGAA 177
CCATGAGGGT 277 115114167 0.466860073 6 0.141087 AGACACAAAA GTTTGATGAA
AGACACAAAA AGGAGGAGA ATGG GGGCCTGGA 178 CCGGTGTCTC 278 115112551
0.46665298 7 0.01555 TTCCACGGCC GGGCCTGGA A TTCCACGGCC ATGGTGGCCT
CTGC CACCCGGACA 179 GATGTACATT 279 115118872 0.462439623 2 0.095238
GCCCCGCTAC CACCCGGACA GCCCCGCTAC TGGTGGGGA ACAG TGAAGAACTG 180
GGGTGCCCG 280 115112219 0.453772064 7 0 TTGGCCCGCC TTGAAGAACT
GTTGGCCCG CCAGGAGGC CCGGGG GGCGGGGCT 181 ACGAGATGGT 281 115112439
0.45003226 7 0 ATGGCGTGAG GGCGGGGCT T ATGGCGTGAG TCGGCGGGC GACGC
CCCGGGGCG 182 GCCCGCCAG 282 115112242 0.446022564 7 0 AAGCCGAGG
GCCCGGGGC CC GAAGCCGAG GCCAGGAGG CAGGGGG GCCCTGGGC 183
CAGGCAGGG 283 115112271 0.440747673 7 0 CAGGTGCGC GGCCCTGGG GG
CCAGGTGCG CGGCGGCGG CCGCGTC TGGCTCTTAC 184 AAGATGTCAT 284 115117421
0.440350741 4 0.237981 AATGTGGAAC TGGCTCTTAC AATGTGGAAC CGGCGGGGC
TGGT TGTTGGTGAG 185 TCTGAAATGT 285 115118726 0.437592504 2 0.289593
TTTCAGTTTG TGTTGGTGAG TTTCAGTTTG TGGTGGAAAG TGA TCGGTGGATG 186

TTCACAATTC 286 115118817 0.43734768 2 0.083612 GTGGCTGGTA TCGGTGGATG GTGGCTGGTA AGGAGGCCG ACCC AGGCCATGG 187 GCAGCGGCA 287 115112072 0.436752641 7 0 CCGTGGAATC GAGGCCATG C GCCGTGGAAT CCAGGAGGC CCGAGA GGGTGAATGT 188 GGGGCTGTC 288 115118797 0.434996943 2 0 ACATCCTCTT CGGGTGAATG TACATCCTCT TTGGTGGCAT TTCG TCCCTGCGCC 189 CCCCGGACA 289 115112052 0.433714562 7 0.041663 GCAGCGGCA GTCCCTGCGC G CGCAGCGGC AGAGGAGGC CATGGC GGTTCGCGA 190 GCAGGAGCC 290 115112260 0.432583783 7 0 GGGCACAGC CGGTTCGCGA GC GGGCACAGC GCCGGCGGC CAAGGT GGAGATCTTG 191 ACGTGGTGGT 291 115112571 0.425267921 7 0 GCCGCGTCG GGAGATCTTG C GCCGCGTCG CAGGAGGCC TCGGG TGGCAAACTG 192 GCGCCCCCC 292 115112180 0.421342539 7 0.045918 GCTGGGGTG ATGGCAAACT C GGCTGGGGT GCAGGAGGA AGAGCG ACGAGATGGT 193 CGAGTGCTG 293 115112429 0.418222381 7 0 GGCGGGGCT GACGAGATG A GTGGCGGGG CTATGGTGGC GTGAGT TCCTGCGCTC 194 CGAACCGGG 294 115112393 0.415381631 7 0.047431 CTCCGCGCC CTCCTGCGCT C CCTCCGCGC CCAGGAGGC CGCGAG AACTGTTGGC 195 CCCGTTGAAG 295 115112224 0.413331832 7 0 CCGCCAGGC AACTGTTGGC C CCGCCAGGC CCGGCGGGG CGAAG TATGTCCGAA 196 GGGGAACAA 296 115117381 0.411925573 4 0 ATGTACTATA GTATGTCCGA AATGTACTAT AAGGAGGGA GTTTC TGTCCAGCCG 197 GGCGACGCTT 297 115112471 0.404128254 7 0.049451 CCCGCTGTCC TGTCCAGCCG CCCGCTGTCC CGGCGGGGC CGCT AGGCGGCTG 198 CCTACAGTGG 298 115114164 0.391417195 6 0.024913 GAGAAGAAG AGGCGGCTG CC GAGAAGAAGC CTGGTGGGC GAAGC CGCGCCTCTT 199 CGGGAGCGC 299 115112347 0.384325854 7 0.051996 CCACCTTGGC GCGCGCCTCT TCCACCTTGG CCGGCGGCG CTGTG CTCCACCGGT 200 TTTCTGGGGC 300 115112536 0.379428798 7 0 GTCTCGGGC CTCCACCGGT C GTCTCGGGC CTGGTGGATT CCAC TGGGGCCTC 201 CCACGGTTTC 301 115112530 0.375527286 7 0 CACCGGTGTC TGGGGCCTC T CACCGGTGTC TCGGCGGGC CTGGA GTTATAAATTT 202 GATGACTGTC 302 115118798 0.368720329 2 0.015571 CACAATTCT GTTATAAATTT CACAATTCTC GGCGGTGGA TGG GGCGTCGCT 203 TCTCGGCCTC 303 115112628 0.360741554 7 0 CTCACCCTCG GGCGTCGCT C CTCACCCTCG CTGGTGGGA CATAA GGGGCTGCC 204 CCTTGACCGC 304 115112529 0.357111718 7 0 CTTGTCACGG GGGGCTGCC C CTTGTCACGG CAGGAGGGC TCCTC TCCCCAGTAG 205 CATCCACTGT 305 115118776 0.356138987 2 0.11655 CGGGGCTGT TCCCCAGTAG C CGGGGCTGT CCGGCGGGT GAATG AACTCCCTTA 206 GACATCTTGA 306 115117396 0.339348533 4 0 TAGTACATTT AACTCCCTTA TAGTACATTT CGGCGGACA TACT CGCCGCAGC 207 ACAGTCCCTG 307 115112058 0.319953253 7 0 GGCAGAGGC CGCCGCAGC CA GGCAGAGGC CATGGTGGCC GTGGA CACAGCGATG 208 GGTATGCAGT 308 115117350 0.30653678 4 0 AATTCAGTTT CACAGCGATG AATTCAGTTT CGGCGGGGA ACAA GCTGCGGCG 209 GGCCTCTGCC 309 115112582 0.304673453 7 0 CAGGGACTGT GCTGCGGCG C CAGGGACTGT CCGGCGGGG CGTCC CTCGTCCAGC 210 CCGCCACCAT 310 115112215 0.299666415 7 0 ACTCGCGGC CTCGTCCAGC C ACTCGCGGC CTGGTGGGC GCGGA CTTGGCCGC 211 TGGTGGAGAT 311 115112577 0.297388466 7 0 GTCGCAGGC CTTGGCCGC CT GTCGCAGGC CTCGGCGGG GCCATG GGCGCTGTG 212 CACCTTGGCC 312 115112368 0.28490812 7 0.039409 CCCTCGCGAA GGCGCTGTG C CCCTCGCGAA CCGGCGGGC TCCTG CCAGAACATC 213 TTTATCCAGC 313 115118935 0.276653116 2 0 TCACTTTAAA CCAGAACATC TCACTTTAAAT GGTGGAGGA AAC GTCCATCAAT 214 CAGCTATAAT 314 115118898 0.268535242 2 0 AAAATGTATT GTCCATCAAT AAAATGTATTT GGTGGCTTTT TT GTACATCCTC 215 CCGGGTGAAT 315 115118805 0.252451409 2 0 TTTGGCATTT GTACATCCTC TTTGGCATTT CGGCGGGGT CGGC CCATTTAAAG 216 AATGTTTCCT 316 115118716 0.216921734 2 0 TGAGATGTTC CCATTTAAAG TGAGATGTTC TGGTGGGCT GGAT

CCCCTCCTGG 217 TGGCATGGGT 317 115112510 − 7 0 CCACGGTTTC CCCCTCCTGG 0.030230013 CCACGGTTTC TGGTGGGGC CTCC
(b) Pharmaceutical Compositions
[0080] The siRNA molecules targeting TBX3 disclosed herein for use according to the methods described herein may be provided per se and/or as part of a pharmaceutical composition, where modulators and/or inhibitors can be mixed with suitable carriers or excipients. As used herein a “pharmaceutical composition” refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.
[0081] Herein the term “active ingredient” refers to any of the siRNA molecules. The term “active ingredient” as used herein can also include any vector, medium, microorganism, or cell culture wherein the siRNA molecule is synthesized, expressed and/or contained, such as a genetically modified cell, viral vector, plasmid, bacteria, yeast, fungus, and the culture or medium thereof.
(i) Pharmaceutically Acceptable Carriers and Excipients
[0082] Hereinafter, the phrases “physiologically acceptable carrier” and “pharmaceutically acceptable carrier” are interchangeably used herein to refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.
[0083] In certain embodiments, compositions disclosed herein may further compromise one or more pharmaceutically acceptable diluent(s), excipient(s), and/or carrier(s). As used herein, a pharmaceutically acceptable diluent, excipient, or carrier, refers to a material suitable for administration to a subject without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained. Pharmaceutically acceptable diluents, carriers, and excipients can include, but are not limited to, physiological saline, Ringer's solution, phosphate solution or buffer, buffered saline, and other carriers known in the art.
[0084] In some embodiments, pharmaceutical compositions herein may also include stabilizers, anti-oxidants, colorants, other medicinal or pharmaceutical agents, carriers, adjuvants, preserving agents, stabilizing agents, wetting agents, emulsifying agents, solution promoters, salts, solubilizers, antifoaming agents, antioxidants, dispersing agents, surfactants, or any combination thereof. Herein, the term “excipient” refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols. Techniques for formulation and administration of drugs may be found in “Remington's Pharmaceutical Sciences,” Mack Publishing Co., Easton, Pa., latest edition, which is incorporated herein by reference.
[0085] In certain embodiments, pharmaceutical compositions described herein may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries to facilitate processing of genetically modified endothelial progenitor cells into preparations which can be used pharmaceutically. In some embodiments, any of the well-known techniques, carriers, and excipients may be used as suitable and/or as understood in the art.
[0086] In certain embodiments, pharmaceutical compositions described herein may be an aqueous suspension comprising one or more polymers as suspending agents. In some embodiments, polymers that may comprise pharmaceutical compositions described herein include: water-soluble polymers such as cellulosic polymers, e.g., hydroxypropyl methylcellulose; water-insoluble polymers such as cross-linked carboxyl-containing polymers; mucoadhesive polymers, selected from, for example, carboxymethylcellulose, carbomer (acrylic acid polymer), poly(methylmethacrylate), polyacrylamide, polycarbophil, acrylic acid/butyl acrylate copolymer, sodium alginate, and dextran; or a combination thereof. In some embodiments, pharmaceutical

compositions disclosed herein may comprise at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50% total amount of polymers as suspending agent(s) by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of polymers as suspending agent(s) by total weight of the composition.

[0087] In certain embodiments, pharmaceutical compositions disclosed herein may comprise a viscous formulation. In some embodiments, viscosity of composition herein may be increased by the addition of one or more gelling or thickening agents. In some embodiments, compositions disclosed herein may comprise one or more gelling or thickening agents in an amount to provide a sufficiently viscous formulation to remain on treated tissue. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50% total amount of gelling or thickening agent(s) by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of gelling or thickening agent(s) by total weight of the composition. In some embodiments, suitable thickening agents for use herein can be hydroxypropyl methylcellulose, hydroxyethyl cellulose, polyvinylpyrrolidone, carboxymethyl cellulose, polyvinyl alcohol, sodium chondroitin sulfate, sodium hyaluronate. In other aspects, viscosity enhancing agents can be acacia (gum arabic), agar, aluminum magnesium silicate, sodium alginate, sodium stearate, bladderwrack, bentonite, carbomer, carrageenan, Carbopol, xanthan, cellulose, microcrystalline cellulose (MCC), ceratonia, chitin, carboxymethylated chitosan, chondrus, dextrose, furcellaran, gelatin, Ghatti gum, guar gum, hectorite, lactose, sucrose, maltodextrin, mannitol, sorbitol, honey, maize starch, wheat starch, rice starch, potato starch, gelatin, sterculia gum, xanthum gum, gum tragacanth, ethyl cellulose, ethylhydroxyethyl cellulose, ethylmethyl cellulose, methyl cellulose, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl cellulose, poly(hydroxyethyl methacrylate), oxypolygelatin, pectin, polygeline, povidone, propylene carbonate, methyl vinyl ether/maleic anhydride copolymer (PVM/MA), poly(methoxyethyl methacrylate), poly(methoxyethoxyethyl methacrylate), hydroxypropyl cellulose, hydroxypropylmethyl-cellulose (HPMC), sodium carboxymethyl-cellulose (CMC), silicon dioxide, polyvinylpyrrolidone (PVP: povidone), Splenda (dextrose, maltodextrin and sucralose), or any combination thereof.

[0088] In certain embodiments, pharmaceutical compositions disclosed herein may comprise additional agents or additives selected from a group including surface-active agents, detergents, solvents, acidifying agents, alkalizing agents, buffering agents, tonicity modifying agents, ionic additives effective to increase the ionic strength of the solution, antimicrobial agents, antibiotic agents, antifungal agents, antioxidants, preservatives, electrolytes, antifoaming agents, oils, stabilizers, enhancing agents, and the like. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50% total amount of one or more agents by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more agents by total weight of the composition. In some embodiments, one or more of these agents may be added to improve the performance, efficacy, safety, shelf-life and/or other property of the muscarinic antagonist composition of the present disclosure. In some embodiments, additives may be biocompatible, without being harsh, abrasive, and/or allergenic.

[0089] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more acidifying agents. As used herein, “acidifying agents” refers to compounds used to provide an acidic medium. Such compounds include, by way of example and without limitation, acetic acid,

amino acid, citric acid, fumaric acid and other alpha hydroxy acids, such as hydrochloric acid, ascorbic acid, and nitric acid and others known to those of ordinary skill in the art. In some embodiments, any pharmaceutically acceptable organic or inorganic acid may be used. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more acidifying agents by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more acidifying agents by total weight of the composition.

[0090] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more alkalizing agents. As used herein, “alkalizing agents” are compounds used to provide alkaline medium. Such compounds include, by way of example and without limitation, ammonia solution, ammonium carbonate, diethanolamine, monoethanolamine, potassium hydroxide, sodium borate, sodium carbonate, sodium bicarbonate, sodium hydroxide, triethanolamine, and trolamine and others known to those of ordinary skill in the art. In some embodiments, any pharmaceutically acceptable organic or inorganic base can be used. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more alkalizing agents by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more alkalizing agents by total weight of the composition.

[0091] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more antioxidants. As used herein, “antioxidants” are agents that inhibit oxidation and thus can be used to prevent the deterioration of preparations by the oxidative process. Such compounds include, by way of example and without limitation, ascorbic acid, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium ascorbate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite and other materials known to one of ordinary skill in the art. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more antioxidants by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more antioxidants by total weight of the composition.

[0092] In certain embodiments, pharmaceutical compositions disclosed herein may comprise a buffer system. As used herein, a “buffer system” is a composition comprised of one or more buffering agents wherein “buffering agents” are compounds used to resist change in pH upon dilution or addition of acid or alkali. Buffering agents include, by way of example and without limitation, potassium metaphosphate, potassium phosphate, monobasic sodium acetate and sodium citrate anhydrous and dihydrate and other materials known to one of ordinary skill in the art. In some embodiments, any pharmaceutically acceptable organic or inorganic buffer can be used. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more buffering agents by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more buffering agents by total weight of the composition.

[0093] In some embodiments, the amount of one or more buffering agents may depend on the desired pH level of a composition. In some embodiments, pharmaceutical compositions disclosed

herein may have a pH of about 6 to about 9. In some embodiments, pharmaceutical compositions disclosed herein may have a pH greater than about 8, greater than about 7.5, greater than about 7, greater than about 6.5, or greater than about 6.

[0094] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more preservatives. As used herein, “preservatives” refers to agents or combination of agents that inhibits, reduces or eliminates bacterial growth in a pharmaceutical dosage form. Non-limiting examples of preservatives include Nipagin, Nipasol, isopropyl alcohol and a combination thereof. In some embodiments, any pharmaceutically acceptable preservative can be used. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more preservatives by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more preservatives by total weight of the composition.

[0095] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more surface-acting reagents or detergents. In some embodiments, surface-acting reagents or detergents may be synthetic, natural, or semi-synthetic. In some embodiments, compositions disclosed herein may comprise anionic detergents, cationic detergents, zwitterionic detergents, ampholytic detergents, amphoteric detergents, nonionic detergents having a steroid skeleton, or a combination thereof. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more surface-acting reagents or detergents by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more surface-acting reagents or detergents by total weight of the composition.

[0096] In certain embodiments, pharmaceutical compositions disclosed herein may comprise one or more stabilizers. As used herein, a “stabilizer” refers to a compound used to stabilize an active agent against physical, chemical, or biochemical process that would otherwise reduce the therapeutic activity of the agent. Suitable stabilizers include, by way of example and without limitation, succinic anhydride, albumin, sialic acid, creatinine, glycine and other amino acids, niacinamide, sodium acetyltryptophanate, zinc oxide, sucrose, glucose, lactose, sorbitol, mannitol, glycerol, polyethylene glycols, sodium caprylate and sodium saccharin and others known to those of ordinary skill in the art. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more stabilizers by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more stabilizers by total weight of the composition.

[0097] In some embodiments, pharmaceutical compositions disclosed herein may comprise one or more tonicity agents. As used herein, a “tonicity agents” refers to a compound that can be used to adjust the tonicity of the liquid formulation. Suitable tonicity agents include, but are not limited to, glycerin, lactose, mannitol, dextrose, sodium chloride, sodium sulfate, sorbitol, trehalose and others known to those or ordinary skill in the art. Osmolarity in a composition may be expressed in milliosmoles per liter (mOsm/L). Osmolarity may be measured using methods commonly known in the art. In some embodiments, a vapor pressure depression method is used to calculate the osmolarity of the compositions disclosed herein. In some embodiments, the amount of one or more tonicity agents comprising a pharmaceutical composition disclosed herein may result in a composition osmolarity of about 150 mOsm/L to about 500 mOsm/L, about 250 mOsm/L to about

500 mOsm/L, about 250 mOsm/L to about 350 mOsm/L, about 280 mOsm/L to about 370 mOsm/L or about 250 mOsm/L to about 320 mOsm/L. In some embodiments, a composition herein may have an osmolality ranging from about 100 mOsm/kg to about 1000 mOsm/kg, from about 200 mOsm/kg to about 800 mOsm/kg, from about 250 mOsm/kg to about 500 mOsm/kg, or from about 250 mOsm/kg to about 320 mOsm/kg, or from about 250 mOsm/kg to about 350 mOsm/kg or from about 280 mOsm/kg to about 320 mOsm/kg. In some embodiments, a pharmaceutical composition described herein may have an osmolarity of about 100 mOsm/L to about 1000 mOsm/L, about 200 mOsm/L to about 800 mOsm/L, about 250 mOsm/L to about 500 mOsm/L, about 250 mOsm/L to about 350 mOsm/L, about 250 mOsm/L to about 320 mOsm/L, or about 280 mOsm/L to about 320 mOsm/L. In some embodiments, pharmaceutical compositions disclosed herein may comprise at least about 5%, at least about 10%, 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% total amount of one or more tonicity modifiers by total weight of the composition. In some embodiments, pharmaceutical compositions disclosed herein may comprise about 5% to about 99%, about 10%, about 95%, or about 15% to about 90% total amount of one or more tonicity modifiers by total weight of the composition.

(ii) Dosage Formulations

[0098] In certain embodiments, the present disclosure provides compositions formulated for one or more routes of administration. Suitable routes of administration may, for example, include oral, rectal, transmucosal, transnasal, intestinal, and/or parenteral delivery. In some embodiments, compositions herein formulated can be formulated for parenteral delivery. In some embodiments, compositions herein formulated can be formulated intramuscular, subcutaneous, intramedullary, intravenous, intraperitoneal, and/or intranasal injections.

[0099] In certain embodiments, one may administer a composition herein in a local or systemic manner, for example, via local injection of the pharmaceutical composition directly into a tissue region of a patient. In some embodiments, a pharmaceutical composition disclosed herein can be administered parenterally, e.g., by intravenous injection, intracerebroventricular injection, intra-cisterna magna injection, intra-parenchymal injection, or a combination thereof. In some embodiments, a pharmaceutical composition disclosed herein can administered to subject as disclosed herein. In some embodiments, a pharmaceutical composition disclosed herein can administered to human patient. In some embodiments, a pharmaceutical composition disclosed herein can administered to a human patient via at least two administration routes. In some embodiments, the combination of administration routes by be intracerebroventricular injection and intravenous injection; intrathecal injection and intravenous injection; intra-cisterna magna injection and intravenous injection; and/or intra-parenchymal injection and intravenous injection.

[0100] In certain embodiments, pharmaceutical compositions of the present disclosure may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0101] In certain embodiments, pharmaceutical compositions for use in accordance with the present disclosure thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. For injection, the active ingredients of a pharmaceutical composition herein may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, physiological salt buffer, or any combination thereof.

[0102] In certain embodiments, pharmaceutical compositions described herein may be formulated in the form of a nanoparticle. The nanoparticle may have a monolayer enclosing the nanoparticle core, wherein the siRNA molecule is disposed within the nanoparticle core. In an embodiment, the

nanoparticle core includes a solid lipid (i.e., lipid that remains solid at room temperature and body temperature) or a liquid lipid (i.e., oil, which remains liquid at room temperature and body temperature, for example, vegetable oil or a lipid extracted from human adipose tissue). In particular, embodiments of the present disclosure include nanoparticles and compositions for the controlled and/or sustained release (e.g., release at a predetermined rate to maintain a certain concentration for a certain period of time) of an agent, such as a small interfering RNA (siRNA) from the nanoparticle.

[0103] In certain embodiments, pharmaceutical compositions described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection herein may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. In some embodiments, compositions herein may be suspensions, solutions or emulsions in oily or aqueous vehicles, and/or may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0104] In certain embodiments, pharmaceutical compositions herein formulated for parenteral administration may include aqueous solutions of the active preparation (e.g., a siRNA molecule) in water-soluble form. In some embodiments, compositions herein comprising suspensions of the active preparation may be prepared as oily or water-based injection suspensions. Suitable lipophilic solvents and/or vehicles for use herein may include, but are not limited to, fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. In some embodiments, compositions herein comprising aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, and/or dextran. In some embodiments, compositions herein comprising a suspension may also contain one or more suitable stabilizers and/or agents which increase the solubility of the active ingredients (e.g., a siRNA molecule) to allow for the preparation of highly concentrated solutions.

[0105] In some embodiments, compositions herein may comprise the active ingredient in a powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water-based solution, before use.

[0106] Pharmaceutical compositions suitable for use in context of the present disclosure may include compositions wherein the active ingredients can be contained in an amount effective to achieve the intended purpose. In some embodiments, a therapeutically effective amount means an amount of active ingredients (e.g., a siRNA molecule) effective to prevent, slow, alleviate or ameliorate symptoms of a disorder (e.g., lymphoproliferative disorders, lymphoid malignancy) or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. For any preparation used in the methods of the present disclosure, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays and or screening platforms disclosed herein. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

[0107] In some embodiments, toxicity and therapeutic efficacy of the active ingredients disclosed herein (e.g., a siRNA molecule) can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. In some embodiments, data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in a human subject. In some embodiments, a dosage for use herein may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in “The Pharmacological Basis of Therapeutics”, Ch. 1).

[0108] In certain embodiments, dosage amounts and/or dosing intervals may be adjusted individually to brain or blood levels of the active ingredient that are sufficient to induce or suppress

the biological effect (minimal effective concentration, MEC). In some embodiments, the MEC for an active ingredient (e.g., a siRNA molecule or composition disclosed herein) may vary for each preparation but can be estimated from in vitro data. In some embodiments, dosages necessary to achieve the MEC herein may depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

[0109] In certain embodiments, depending on the severity and responsiveness of the condition to be treated, dosing with compositions herein can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

[0110] In certain embodiments, amounts of a composition herein to be administered will be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, and the like. In some embodiments, effective doses may be extrapolated from dose-responsive curves derived from in vitro or in vivo test systems.

III. Methods

[0111] The present disclosure provides for methods of treating, attenuating, and preventing liver disease in a subject in need thereof. In several embodiments, a method for treating, attenuating, or preventing liver disease in a subject can include administering to a subject, including a human subject, an effective amount of one or more siRNA molecules targeting TBX3 disclosed herein or a nucleic acid encoding an siRNA molecule targeting TBX3 as disclosed herein. In several embodiments, a method for treating, attenuating, or preventing liver disease in a subject can include administering to a subject, including a human subject, an effective amount of a nucleic acid encoding a suitable sgRNA, or a suitable sgRNA targeting TBX3 and an RNA guided endonuclease.

[0112] Methods disclosed herein may include treating a subject in need thereof by administrating a therapeutically effective amount of one or more siRNA molecules or a pharmaceutical composition disclosed herein. The subject may be a human subject having or suspected of having, or at risk of having liver disease, liver damage, liver dysfunction, liver injury. The term “liver disease”, “liver injury” or “liver dysfunction” may be used interchangeably and refer to any injury of the liver, including but not limited to hardening of the liver, scarring of the liver, decreased or abnormal biliary tract function, abnormal liver enzyme activity, cirrhosis of the liver, abnormal physiology as determined by common diagnostic methods include but not limited to ultrasound, or biopsy/histopathology, necrosis of the liver and the like. Non-limiting examples of liver disease to be treated using the methods disclosed herein may include fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer HCC, or any combination thereof. In some embodiments, the subject benefits of an increase in life expectancy compared to an untreated subject with identical disease condition and predicted outcome. In some other embodiments, the treatment improves the subject's liver function as compared to an untreated subject with identical disease condition and predicted outcome. In yet some other embodiments, the treatment attenuates the subject's liver fibrosis as compared to an untreated subject with identical disease condition and predicted outcome. In some embodiments, the treatment prevents additional liver fibrosis in the subject compared to an untreated subject with identical disease condition and predicted outcome.

[0113] A subject suitable for the liver disease treatment as disclosed herein may be selected based on the subject's diagnosis. In some embodiments, a method of diagnosis may detect one or more serum markers indicative of liver disease. Non-limiting examples of serum markers indicative of a liver disease (e.g., NAFLD, NASH, or HCC) may include alpha-fetoprotein (AFP) (e.g., an AFP level of 20 ng/mL or higher), des-gamma-carboxy prothrombin, lens culinaris agglutinin-reactive AFP (AFP-L3), and the like. The diagnosis method may also include the evaluation of at least one clinical symptom associated with a liver disease. Non-limiting examples of clinical symptoms

associated with a liver disease may include mild to moderate upper abdominal pain, weight loss, early satiety, or a palpable mass in the upper abdomen, paraneoplastic syndrome, hypoglycemia, erythrocytosis, hypercalcemia, intractable diarrhea and associated electrolyte disturbances (e.g., hyponatremia, hypokalemia, metabolic alkalosis), cutaneous manifestations (e.g., dermatomyositis, pemphigus foliaceus, seborrheic keratosis, pityriasis rotunda), intraperitoneal bleeding, jaundice, fever, pyogenic liver abscess, and the like. Other aspects of diagnosis may include at diagnosis and/or a determination of severity of cirrhosis, chronic infection of hepatitis B virus (HBV), chronic infection of hepatitis C virus (HCV), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis (PBC), hereditary hemochromatosis, type 2 diabetes, obesity, tobacco use, alcohol abuse, long-term anabolic steroid use, tyrosinemia, alpha1-antitrypsin deficiency, porphyria cutanea tarda, glycogen storage diseases, Wilson disease, or any combination thereof.

[0114] In some embodiments, a subject can be diagnosed and/or predicted to have high or low risk for a liver disease (e.g., NASH or NAFLD) by histological or imaging-based examinations, such as contrast-enhanced multiphase CT, ultrasound, and/or MRI. Imaging features used to diagnose may include liver size, kinetics, and pattern of contrast enhancement, and growth on serial imaging wherein size may be measured as the maximum cross-section diameter on the image where the lesion is most clearly seen. The histologic appearance of NASH or NAFLD biopsies can include steatosis, inflammation, and fibrosis.

[0115] The methods and compositions of the present disclosure are useful for the treatment of subjects having fatty liver related disorders, such as NAFLD and/or NASH. The subject may have normal or substantially normal biliary tract function. Normal or substantially normal biliary tract function may be determined in a subject using any suitable methods known in the art. Generally, preferred tests for biliary tract function in NASH patients may be characterized in two groups: physiological based tests and biochemical based tests. Physiological based tests may include but are not limited to abdominal ultrasound, abdominal CT scan, endoscopic retrograde cholangiopancreatography (ECRP), Percutaneous transhepatic cholangiogram (PTCA) or Magnetic resonance cholangiopancreatography (MRCP). Biochemical based tests may include but are not limited to GGT tests, liver function tests, bilirubin tests, alkaline phosphatase (ALP) tests, liver enzyme tests, amylase blood test, lipase blood test, prothrombin time, and measurement of urine bilirubin. In some cases, one or more tests may be used to characterize biliary function. In some cases, a combination of tests may be used to assess biliary function in NASH subjects.

[0116] In some embodiments, any of the methods disclosed herein can further include monitoring for an occurrence of one or more adverse effects in the subject. Adverse effects may include, but are not limited to, hepatic impairment, hematologic toxicity, neurologic toxicity, cutaneous toxicity, gastrointestinal toxicity, or a combination thereof. When one or more adverse effects are observed, the methods disclosed herein can further include reducing or increasing the dose of one or more of the treatment regimens depending on the adverse effect or effects in the subject. For example, when a moderate to severe hepatic impairment is observed in a subject after treatment, compositions of use to treat the subject can be reduced in concentration or frequency.

[0117] In certain embodiments, treatments administered according to the methods disclosed herein can improve patient life expectancy compared to the life expectancy of an untreated subject with identical disease condition (e.g., NAFLD or NASH) and predicted outcome. As used herein, “patient life expectancy” is defined as the time at which 50 percent of subjects are alive and 50 percent have passed away. In some embodiments, patient life expectancy can be indefinite following treatment according to the methods disclosed herein. In other aspects, patient life expectancy can be increased at least about 5% or greater to at least about 100%, at least about 10% or greater to at least about 95% or greater, at least about 20% or greater to at least about 80% or greater, at least about 40% or greater to at least about 60% or greater compared to an untreated subject with identical disease condition and predicted outcome. In some embodiments, patient life

expectancy can be increased at least about 5% or greater, at least about 10% or greater, at least about 15% or greater, at least about 20% or greater, at least about 25% or greater, at least about 30% or greater, at least about 35% or greater, at least about 40% or greater, at least about 45% or greater, at least about 50% or greater, at least about 55% or greater, at least about 60% or greater, at least about 65% or greater, at least about 70% or greater, at least about 75% or greater, at least about 80% or greater, at least about 85% or greater, at least about 90% or greater, at least about 95% or greater, at least about 100% compared to an untreated subject with identical disease condition and predicted outcome. In some embodiments, patient life expectancy can be increased at least about 5% or greater to at least about 10% or greater, at least about 10% or greater to at least about 15% or greater, at least about 15% or greater to at least about 20% or greater, at least about 20% or greater to at least about 25% or greater, at least about 25% or greater to at least about 30% or greater, at least about 30% or greater to at least about 35% or greater, at least about 35% or greater to at least about 40% or greater, at least about 40% or greater to at least about 45% or greater, at least about 45% or greater to at least about 50% or greater, at least about 50% or greater to at least about 55% or greater, at least about 55% or greater to at least about 60% or greater, at least about 60% or greater to at least about 65% or greater, at least about 65% or greater to at least about 70% or greater, at least about 70% or greater to at least about 75% or greater, at least about 75% or greater to at least about 80% or greater, at least about 80% or greater to at least about 85% or greater, at least about 85% or greater to at least about 90% or greater, at least about 90% or greater to at least about 95% or greater, at least about 95% or greater to at least about 100% compared to an untreated patient with identical disease condition and predicted outcome.

[0118] In some embodiments, treatment of a liver disease, such as NAFLD or NASH, according to the methods disclosed herein can result in an attenuating, a shrinking, a reducing or a preventing of a liver fibrosis in comparison to the starting size of the liver fibrosis. In some embodiments, liver fibrosis attenuating or shrinking may be at least about 5% or greater to at least about 10% or greater, at least about 10% or greater to at least about 15% or greater, at least about 15% or greater to at least about 20% or greater, at least about 20% or greater to at least about 25% or greater, at least about 25% or greater to at least about 30% or greater, at least about 30% or greater to at least about 35% or greater, at least about 35% or greater to at least about 40% or greater, at least about 40% or greater to at least about 45% or greater, at least about 45% or greater to at least about 50% or greater, at least about 50% or greater to at least about 55% or greater, at least about 55% or greater to at least about 60% or greater, at least about 60% or greater to at least about 65% or greater, at least about 65% or greater to at least about 70% or greater, at least about 70% or greater to at least about 75% or greater, at least about 75% or greater to at least about 80% or greater, at least about 80% or greater to at least about 85% or greater, at least about 85% or greater to at least about 90% or greater, at least about 90% or greater to at least about 95% or greater, at least about 95% or greater to at least about 100% (meaning that the liver fibrosis is completely gone after treatment) compared to the starting size of the liver fibrosis.

[0119] In some embodiments, treatment of a liver disease, such as NAFLD or NASH, according to the methods disclosed herein can result in an improved liver function. Liver function may be tested through routine biochemical methods. Biochemical based tests may include but are not limited to GGT tests, liver function tests, bilirubin tests, alkaline phosphatase (ALP) tests, liver enzyme tests, amylase blood test, lipase blood test, prothrombin time, and measurement of urine bilirubin. In some cases, one or more tests may be used to characterize biliary function. In some cases, a combination of tests may be used to assess biliary functions. The liver function improvement can also be assessed by subject's symptom relief, such as relieving in fatigue, weight loss, and weakness of the subject. Other relieved symptoms may include reduced fluid retention, muscle wasting, bleeding from the intestines, and any combination thereof.

IV. Kits

[0120] The present disclosure provides kits for use in the methods disclosed herein. In some

aspects, the present disclosure provides a kit for treating a liver disease (e.g., NASH or NAFLD) as disclosed herein and/or for diagnosing the liver disease. Such a kit may comprise a means for holding and/or administering such a siRNA composition or a pharmaceutical composition.

[0121] In some embodiments, kits disclosed herein can have a medical container, which holds the composition in a safe, stable and durable way. In some examples, kits disclosed herein may also comprise a means to administer the composition, such as a needle or a spatula.

[0122] Any of the kits may further comprise an instruction manual providing guidance for using the kit for treatment. The manual may be written with the physician or the liver specialist as the intended reader.

[0123] Having described several embodiments, it will be recognized by those skilled in the art that various modifications, alternative constructions, and equivalents may be used without departing from the spirit of the present inventive concept. Additionally, a number of well-known processes and elements have not been described in order to avoid unnecessarily obscuring the present inventive concept. Accordingly, this description should not be taken as limiting the scope of the present inventive concept.

[0124] Those skilled in the art will appreciate that the presently disclosed embodiments teach by way of example and not by limitation. Therefore, the matter contained in this description or shown in the accompanying drawings should be interpreted as illustrative and not in a limiting sense. The following claims are intended to cover all generic and specific features described herein, as well as all statements of the scope of the method and assemblies, which, as a matter of language, might be said to fall there between.

EXAMPLES

[0125] The following examples are included to demonstrate preferred embodiments of the disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the present disclosure, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the present disclosure.

Example 1

[0126] Somatic mutation screening of transcription and epigenetic factors identified therapeutic targets in NASH. It was found that somatic mutations in hepatocytes could be promoting cellular fitness through the suppression of lipotoxicity. Using transcriptomic data from HCV cirrhosis and NASH patients, transcriptional or epigenetic regulators with altered gene expression in these disease states were identified. AAV-sgRNA libraries containing pools of sgRNAs targeting either transcription or epigenetic factors were generated, and these libraries to were used perform two independent somatic mosaic screens in the mouse liver. Each library contained 200-300 genes. After the establishment of somatic mutations in these genes using in vivo expression of Cas9, mice are put on normal chow or western diet/sugar water for up to 6 months. The sgRNAs associated with the most clonal expansion in both of these screens targeted a set of 23 genes (13 from the TF library and 10 from the epifactor library). Among the top hits from this in vivo genetic screen analysis was the T-box transcription factor (TBX3, see FIG. **1**). TBX3 has not been studied in fatty liver disease before.

[0127] To further ascertain which genes from the genetic screens were increasing clonal expansion through an influence on metabolic fitness, conditional knockout mouse models were needed. The CRISPR plus AAV-sgRNA approach described herein was first used to generate liver-wide conditional deletion models. AAV-sgRNAs against GFP or LacZ were implemented as independent control models. High titer AAVs-sgRNAs against Tbx3 were injected into Cas9 expressing mice at 8 weeks of age, then after 2 weeks, mice are given western diet (WD) for three months. For each conditional CRISPR KO model, body weight, liver weight, histology, steatosis, fibrosis, serum tests

(liver function tests, cholesterol, TGs, non-esterified free fatty acids (NEFAs)) are measured. Tbx3 KO mice showed reduced liver weight and liver to body weight ratios compared to controls (FIG. **1**). In addition, TBX3 KO mice showed significantly reduced liver triglycerides. Moreover, there was reduced liver damage and inflammation as measured by AST/ALT and reduced hepatic steatosis on histology (See FIGS. **2**A-**2**F). These genetic data showed that TBX3 was a promising therapeutic target in human NASH.

Example 2

[0128] TBX3 floxed mice were used to more rigorously examine loss of TBX3 in the liver. This knockout experiment allows nearly 100% hepatocyte deletion, which cannot be achieved using CRISPR AAV KO approaches, which are less efficient. Spatial and temporal conditional deletion are achieved with AAV-TBG-Cre, which expresses Cre recombinase only in hepatocytes. Mice are given AAV at 6 weeks and initiated on NASH diets at 8 weeks of age. Tbx3.sup.fl/fl and Tbx3.sup.+/+ mice are given AAV-TBG-Cre (n=15 for each genotype, males and females tested separately). Four groups are compared: Tbx3.sup.+/++AAV-TBG-Cre on normal chow (NC), Tbx3.sup.fl/fl+AAV-Cre on NC, Tbx3.sup.+/++AAV-TBG-Cre on WD, Tbx3.sup.fl/fl+AAV-TBG-Cre on WD. The NC groups are used to rule out phenotypic effects of Tbx3 loss that may arise independently of diet. These rigorous reference standard KO mice are used to determine the extent to which NASH can be prevented. Effects of Tbx3 deletion on liver steatosis, inflammation, and fibrosis after 12 and 24 weeks of NASH diets are characterized. In WT control mice fed WD, hepatic steatosis and inflammation are prominent at 12 weeks, while hepatocyte ballooning and fibrosis (features that are more NASH specific), are observed by 24 weeks.

[0129] NASH progression is characterized. Mice are examined twice weekly when food and sugar water are replenished. The mice are visually inspected for signs of distress/illness. Body weights are recorded once weekly. Survival times are recorded in case of death (unexpected). Liver toxicity is monitored prior to liver harvesting (every 12 weeks) by blood AST, ALT, total bilirubin, albumin, and complete blood counts using the UTSW Molecular Phenotyping Core. Examined biomarkers include plasma and liver triglycerides (TG), non-esterified free fatty acids (NEFA), and cholesterol, all of which are the critical lipid markers of NASH and metabolic syndrome. Livers are photographed and/or sectioned for macro/microscopic evidence of tissue injury, inflammation, and fibrosis. Histology and whole slide imaging are performed using an Axioscan microscope. Oil Red-O and Sirius Red stains are used to examine steatosis and fibrosis, respectively. Livers are examined in a blinded fashion for components of the NASH Activity Score (NAS): steatosis, lobular inflammation, and hepatocyte ballooning. Separately, the degree of fibrosis is quantified. If cancers appear, it is quantified. TBX3 suppression resulted in reduced steatosis (FIGS. **1** and **2**A-**2**F) and inflammation. Since it was possible for steatosis to be reduced without concomitant changes in ballooning or fibrosis, long-term WD feeding is critical to ascertain the distinct pathological components of NASH.

[0130] TBX3 hepatocyte specific KO mice have a strong protective effect against steatosis and inflammation than the prior CRISPR KO mice because they have a more complete deletion of TBX3 in more hepatocytes. Over 12 and 24 weeks, it is reasonable to expect a delay, but not complete elimination of steatosis, inflammation, ballooning, and fibrosis.

[0131] To summarize, one of the most promising genetic hits was Tbx3, a gene not known to be involved in NASH prior to this disclosure. Liver specific conditional knockout mouse models were generated to further study Tbx3 in NASH. A CRISPR plus AAV-sgRNA approach was used to generate a liver-wide conditional deletion model for Tbx3 and AAV-sgRNAs against GFP or LacZ were used as independent controls (FIG. **1**). In the context of 3 months of NASH-inducing diets, deletion of Tbx3 showed significantly reduced liver to body weight ratios (FIG. **1**), a highly specific biomarker of liver fat accumulation and NASH. Moreover, there was reduced liver damage and inflammation as measured by AST/ALT, and significantly reduced hepatic steatosis on histology (FIGS. **2**A-**2**F).

Example 3

[0132] In order to address if a mouse siRNA against TBX3 prevents and/or reverses NASH in a mouse model, an siRNA tool compound used in mice is used to study translational aspects of TBX3 biology. Potent siRNA sequences were identified. Next, dosing is optimized to maximize effectiveness in vivo. Eight siRNAs have been tested against mouse TBX3 in Cos7 cells to identify the siRNA sequence with the highest knockdown efficiency based on mouse Tbx3 reporter assays. Then this siRNA is modified and conjugated with GalNAc in the standard fashion (Foster et al., 2018). Briefly, in order to minimize nucleolytic degradation and immune responses in vivo, every 2′-position is modified with 2′-O-methyl. To determine if these stabilizing modifications influence silencing activities, siRNA activity in vitro prior to GalNAc conjugation is determined. In accord with the industry standard approach, GalNAc conjugation allows for high efficiency delivery to hepatocytes without lipid nanoparticle packaging. The best in vivo dosing regimen is then be determined for GalNAc-siTBX3. Three doses of siRNAs in PBS (1, 5, 10, 15 mg/kg) are given subcutaneously (SC), once every two weeks starting at 4 weeks of age to WT B6 mice. After siRNA treatments, mice are sacrificed for TBX3 qPCR, western blotting, and histology analysis (n=5 mice/group). Maximum tolerated dose is determined based on lack of severe toxicities such as weight loss greater than 10%, organ (heart/kidney/liver) failure, and death. In all of the following siRNA experiments, GalNAc-siLuc serves as the control.

[0133] To determine if GalNAc-siTbx3 prevents NASH, the optimized dose defined above is used. Then it is determined if GalNAc-siRNA can mimic the TBX3 KO model. In a parallel fashion as the genetic experiments, GalNAc-siRNA SC injections are started at 6 weeks of age and NASH diets are started at 8 weeks and continued for 12 weeks (6 total siRNA doses). Mice are euthanized at 20 weeks of age, a time point at which steatosis, inflammation, and fibrosis are accessed. The siRNA approach closely mimics the genetic NASH prevention model.

[0134] To determine if NASH can be reversed by GalNAc-siTBX3, siRNA dosing is initiated in mice that have already received 24 weeks of WD diets (30 weeks of age) and the WD and siRNAs are continued for 12 weeks total. Then the livers are assessed for pathological features of NASH. Assessment includes hepatocyte ballooning, inflammation, and fibrosis, features that characterize NASH as described above. These siRNA experiments provide immense translational value and pave the path for clinical therapeutics.

Example 4

[0135] Specific siRNA sequences were designed to target human TBX3 for the treatment of NASH. Effective siRNAs that can knockdown human TBX3 were identified (FIGS. **3**A-**3**B). Tests identified optimized siRNA sequences corresponding to the sequences of human TBX3. This involved screening candidate siRNAs per gene target using in vitro luciferase reporter-based assays. In brief, full length TBX3 cDNA was cloned into a psicheck2 plasmid backbone containing a luciferase gene. This created a *Renilla* luciferase-TBX3 fusion gene. The luciferase assay was a dual reporter system with Firefly luciferase as a control and *Renilla* luciferase as the read out for transcription and translation. Thus, the ratio of *Renilla* to Firefly signal accounted for variations in transfection efficiency and cell viability. The assay was carried out in a 96 well plate format with technical replicates using a 48-hour time point for the assay readout. The reporter along with siRNAs were co-transfected into Cos7 monkey kidney cells using lipofectamine. After 48 hours, the cells were lysed and the signal was captured by a luminometer using the substrates for Firefly and *Renilla*. Effective siRNAs against a target gene showed a reduced ratio for *Renilla* vs. Firefly signal compared to non-targeting controls. A total of 45 siRNAs targeting the entire TBX3 cDNA were designed and tested. siRNAs designed by Dharmacon and ThermoFisher were also tested. At least 15 effective siRNAs were identified against human TBX3 (Table 3). In other words, 15 siRNAs shown in Table 2 had less than 40% of TBX3 mRNA remaining. The siRNAs designed and tested according to the methods disclosed herein shown in FIGS. **3**A-**3**B. Optimized siRNAs are modified by conjugating to acetylgalactosamine (GalNAC) in the standard fashion and tested in

vivo.

TABLE-US-00004 TABLE 3 % of TBX3 mRNA remaining Experimental Design (compared to neg. ctl siRNA, or Manufacturer siRNA ID at 3.3 nM or 0.5 pmol) Design TBX3 470 81.3 Experimental TBX3 1080 22.7 Experimental TBX3 457 83.0 Experimental TBX3 707 85.0 Experimental TBX3 754 89.1 Experimental TBX3 966 79.9 Experimental TBX3 631 81.0 Experimental TBX3 628 77.9 Experimental TBX3 627 76.5 Experimental TBX3 857 77.2 Experimental TBX3 389-411 88.2 Experimental TBX3 390-412 86.7 Experimental TBX3 438-460 82.8 Experimental TBX3 619-641 80.3 Experimental TBX3 768-790 81.2 Experimental TBX3 917-939 127.8 Experimental TBX3 428-450 19.3 Experimental TBX3 437-459 16.5 Experimental TBX3 778-800 73.9 Experimental TBX3 860-882 76.7 Experimental J-012197-05 59.0 Dharmacon J-012197-06 35.3 Dharmacon J-012197-07 40.2 Dharmacon J-012197-08 70.0 Dharmacon D-012197-01 62.7 Dharmacon D-012197-02 20.2 Dharmacon D-012197-03 23.3 Dharmacon D-012197-04 29.3 Dharmacon s13865 26.1 ThermoFisher s13866 21.5 ThermoFisher s13867 14.1 ThermoFisher s529425 82.5 ThermoFisher s534311 18.9 ThermoFisher s534312 27.3 ThermoFisher 107921 50.3 ThermoFisher 107922 54.4 ThermoFisher 115750 59.4 ThermoFisher 115751 66.6 ThermoFisher 139810 126.8 ThermoFisher 239194 30.6 ThermoFisher 239195 64.2 ThermoFisher 239196 55.7 ThermoFisher 3661 36.0 ThermoFisher 3754 43.0 ThermoFisher 3845 31.4 ThermoFisher

[0136] Some of the human siRNAs against TBX3 in Table 2 are tested in human cell line models of NASH. The experimental design has three steps. First, use the identified siRNAs in human liver cancer cells, such as Huh7 or HepG2, to knockdown a target genes of interest, i.e. TBX3. Specifically, Huh7 cells are grown to 60-80% confluence and then transfected with siRNAs. Opti-MEM (Thermo Fisher 31985062) containing the siRNAs and Lipofectamine are combined, incubated for 10 minutes, and then added to cells (400 μl/well to a 6-well plate or 2.4 ml to a 100 mm plate). Transfection medium is replaced after 6 hours with medium containing 100 μM FA. Then, cells are fed lipids and labeled with lipid dyes. Lastly, fluorescence-activated cell sorting (FACS) is performed to purify specific cell populations based on phenotypes detected by flow cytometry. This method enables characterization of a single cell population without the influence of other cells. Cells are stained with 1 ml PBS containing 10 μg of BODIPY® 493/503 (BD) (Thermo Fisher D3922) for 15 minutes at room temperature (23° C.±5° C.), then subjected to FACs measurements. Studies show that in lipophilic fluorophore stained cells, fluorescence intensity measured by FC reflects lipid levels.

[0137] An aliquot of the cells grown under each condition is assayed for triacylglycerol (TG). In brief, cells are trypsinized, washed once with PBS, resuspended in PBS with 10 mM EDTA, and then counted. Cells are divided in triplicate into 13×100 mm glass tubes in a final volume of 200 μl. Triolein standards (Sigma T7140) are also prepared in a final volume of 200 μl PBS/10 mM EDTA in 13×100 mm glass tubes. TGs are extracted and quantified. Briefly, 2 ml of isopropanol:hexane:water (40:10:1) is added to cells or standards and the samples are vortexed, covered, and incubated at room temperature for 30 min. Then, 500 μl of a 1:1 mixture of hexane:diethylether is added to the samples followed by vortexing and incubating for an additional 10 min at room temperature. Next, 1 ml of water is added to samples, tubes are vortexed, and layers are allowed to separate at room temperature while covered for 30-45 min. Using Pasteur pipettes, the top layer is transferred to 12×75 mm glass tubes and dried under N.sub.2 to completion. Following the drying step, 400 μl of Infinity triglyceride reagent (Thermo Scientific TR22421) is added to each tube and vortexed. Tubes are covered and incubated for 90 minutes at 37° C. with shaking at 250 rpm. Finally, 300 μl of each sample is transferred to 96-well plates and absorbance is measured at 540 nm using a microplate reader. To determine whether fixing cells affected the TG measurement, TG is quantified in fixed and unfixed cells from the same batch of Huh7 cells incubated in 2 mM FA.

[0138] The tests described above were performed using optimized siRNAs and/or their conjugates

with acetylgalactosamine (GalNAc) to improve liver targeting.

Example 5

[0139] Deletion of the transcription factor Tbx3 showed significant reductions in body weights (FIG. **4**A) and this deletion resulted in one of the most substantial reductions in liver/body weight ratios compared to controls after 3 months of WD (dark dots in FIG. **4**B). TBX3, a T-box transcription factor, has not been studied in fatty liver disease prior to the current disclosure. The KO model showed the most significant reductions in liver injury as measured by ALT (FIG. **4**C), a trend toward reduced AST (FIG. **4**D), and the most significant reductions in liver triglyceride levels (FIG. **4**E). While Tbx3KO mice had reduced liver cholesterol, Bcl6 KO mice had increased liver cholesterol (FIG. **4**F). Compared to controls and other KO models, there were clear improvements in hepatic steatosis in Tbx3, Bcl6, and Smyd2 KO livers (FIGS. **4**G-**4**I). The phenotypes of Tbx3, Bcl6, and Smyd2 KO mice were comparable to Srebf1 and Dgat2 KO mice, suggesting potent regulation of lipid metabolism by these three genes.

[0140] To examine late-stage NASH features, some CRISPR KO models for 9 months with WD were fed (FIG. **5** and Table 4). In male Tbx3 KO mice, liver weight and liver/body weight ratios decreased, while body weights were unchanged (FIG. **5**A). Significant decreases of ALT and AST were also observed (FIG. **5**B), suggesting reduced liver damage. In male Smyd2 KO mice, liver/body weight ratios decreased, but liver and body weights were unchanged (Figure S**8**A), and ALT/AST levels were unchanged (FIG. **5**B). In the plasma, both Tbx3 and Smyd2 KOs showed moderate increases in triglyceride, but cholesterol levels remained similar to controls (FIG. **5**C). These data showed that both KO models protect the liver from steatosis, but male Tbx3 KO had more pronounced effects under long-term WD (FIG. **5**D). Trends toward decreased fibrosis was observed in both KO models (FIGS. **5**D and **5**E).

TABLE-US-00005 TABLE 4 plasma- plasma- KO body liver liver/body AST ALT Cholesterol Triglyceride # gene sex (gram) (gram) (%) (U/L) (U/L) (mg/dL) (mg/dL) 3671 lacZ M 50.20 6.14 12.23 746 568 488 55.0 3676 lacZ M 40.37 3.77 9.34 347 316 324 70.2 3679 lacZ M 43.12 4.27 9.90 275 290 401 75.4 3680 lacZ M 46.15 4.65 10.08 288 272 427 60.6 3681 lacZ M 51.22 7.30 14.25 339 385 455 54.0 3682 lacZ M 51.62 6.23 12.07 397 382 461 62.9 3683 lacZ M 43.42 4.42 10.18 226 241 407 53.1 3732 smyd2 M 53.12 6.70 12.61 430 439 497 75.7 3737 smyd2 M 49.03 4.58 9.34 386 387 405 74.6 3739 smyd2 M 43.62 3.78 8.67 255 265 343 99.4 3740 smyd2 M 35.32 2.89 8.18 314 171 293 62.3 3741 smyd2 M 44.54 3.97 8.91 406 306 343 72.3 3755 smyd2 M 34.42 2.52 7.32 131 71 229 84.1 3757 smyd2 M 40.72 3.45 8.47 546 380 382 66.7 3758 smyd2 M 47.37 4.95 10.45 442 421 455 82.9 3673 tbx3 M 52.35 5.11 9.76 385 366 503 78.7 3674 tbx3 M 47.19 3.69 7.82 342 268 405 70.9 3687 tbx3 M 41.43 3.33 8.04 123 102 304 105.5 3688 tbx3 M 42.38 3.53 8.33 209 182 358 80.9 3691 tbx3 M 36.77 2.19 5.96 237 113 280 112.1 3692 tbx3 M 39.31 2.57 6.54 234 140 267 68.4 3703 tbx3 M 46.76 3.29 7.04 184 149 316 67.5 3709 tbx3 M 44.52 3.15 7.08 189 150 317 85.1 3730 tbx3 M 50.10 4.71 9.40 399 377 477 105.7 3731 tbx3 M 45.40 3.45 7.60 214 132 273 119.5 3735 tbx3 M 41.92 2.81 6.70 243 166 387 120.7 3738 tbx3 M 45.99 3.79 8.24 Failed to collect 3742 tbx3 M 46.32 3.45 7.45 221 132 353 96.9 3901 gfp F 41.48 3.25 7.84 319 190 275 64.4 3902 gfp F 33.2 2.28 6.87 248 72 172 85.1 3909 gfp F 41.26 2.55 6.18 150 99 252 73.8 3912 gfp F 46.03 4.38 9.52 175 122 159 77.3 3916 gfp F 47.73 5.29 11.08 266 246 376 69.1 3917 gfp F 48.26 4.46 9.24 284 243 306 65.4 3920 gfp F 36.58 2.45 6.70 149 75 245 73.8 3921 gfp F 49.79 4.73 9.50 330 287 404 62.6 3933 gfp F 35.28 2.26 6.41 108 63 126 68.3 3923 smyd2 F 43.72 3.33 7.62 227 154 280 47.5 3924 smyd2 F 43.98 3.95 8.98 302 225 385 72.6 3927 smyd2 F 37.02 2.33 6.29 204 108 307 77.4 3928 smyd2 F 40.72 2.19 5.38 176 114 228 74.8 3929 smyd2 F 36.14 2.46 6.81 250 114 186 55.0 3930 smyd2 F 34.96 1.75 5.01 109 52 152 55.8 3931 smyd2 F 25.57 1.41 5.51 82 36 103 63.9 3932 smyd2 F 42.68 2.72 6.37 164 98 270 74.7 3903 tbx3 F 37.94 2.18 5.75 213 114 220 86.0 3904 tbx3 F 34.08 1.87 5.49 152 34 104 75.0 3908 tbx3 F 41.64 3.12 7.49 173 139 221 92.6 3911 tbx3 F 30.04 1.74 5.79 188 122 121 83.6 3913 tbx3 F 43.43 3.11 7.16 294 183 300 72.5 3914 tbx3 F 46.27 3.56 7.69 331 285 365 82.6 3918 tbx3 F 54.82 4.91

8.96 404 271 365 64.8 3926 tbx3 F 45.53 2.9 6.37 245 213 287 106.4

Example 6

[0141] To investigate the expression changes in WD fed livers carrying fitness promoting mutations, RNA-seq on control (sgGFP, sgLacZ), Irs1, Srebf1, Tbx3, Bcl6, and Smyd2 KO livers generated with MOSAICS AAVs was performed. Comparing differentially regulated genes in control vs. KO livers showed a relatively large intersection between Tbx3 and Irs1 KO livers (FIG. **6**A), suggesting shared regulatory circuits. For lipid metabolism pathways in GSEA analysis, some shared but mostly unique expression patterns for each KO group were observed (FIG. **6**B). The expression of genes involved in lipogenesis and fatty acid oxidation were further examined. Deletion of Irs1, Srebf1, Tbx3, Bcl6, and Smyd2 each led to decreased expression of fatty acid and triglyceride synthesis genes, but to different extents (FIG. **6**C). The mRNA expression of collagen, a major component of fibrosis were also analyzed, and a downregulation of multiple collagen mRNAs in KO livers was observed (FIG. **6**D). These data indicate shared and unique mechanisms by which mutant hepatocytes converge on decreased steatosis and fibrosis.

Experimental Methods and Subjects Used in the Examples

Mouse Strains and Breeding

[0142] All mice were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee at UT Southwestern. All experiments were done in an age and sex controlled fashion unless otherwise noted. All mice used in this study were male. Genotyping and Sanger sequencing was used to confirm homologous recombination in the genome-edited pups. LSL-tdTomato (strain #007914) and Rosa-rtTA; TetO-Cas9 mice (#029415) were obtained from The Jackson Laboratory. Mice homozygous for both Rosa-rtTA and TetO-Cas9 were used to ensure a high Cas9 expression level in the liver. Western Diet (WD) used for NAFLD/NASH modeling is described in. It is composed of high fat solid food (ENVIGO #TD.120528) and high sugar water containing 23.1 g/L d-fructose (Sigma-Aldrich #F0127) and 18.9 g/L d-glucose (Sigma-Aldrich #G8270).

Fluorescent Imaging and Image Processing

[0143] For fluorescent imaging, liver pieces were fixed in buffered formalin (Fisherbrand #245-685) for 24 h with gentle shaking at 4° C. and then transferred into 30% sucrose (w/v) solution for another 24 h with shaking at 4° C. The livers were then embedded and frozen in Cryo-Gel (Leica #39475237), and sectioned at a thickness of 16 μm. Images were taken using a Zeiss Axionscan Z1 system in the UTSW Whole Brain Microscopy Facility to visualize Tomato clones. To statistically analyze the percentage of Tomato+ cells, black and white fluorescent images were taken from the same slide using an Olympus IX83 microscope at 4× magnification. Two different fields were taken for each liver. The percentage of Tomato+ cells (bright areas) was analyzed using ImageJ.

H&E, Immunohistochemistry (IHC), Immunofluorescence (IF), TUNEL, and Sirius Red Staining

[0144] Liver pieces were fixed in buffered formalin (Fisherbrand #245-685) for 24 h with gentle shaking at 4° C. and then transferred to 70% EtOH for another 24 h with shaking at 4° C. Paraffin embedding, liver sectioning (4 μm thickness), and H&E staining were performed at the UT Southwestern Tissue Management Shared Resource Core. IHC was performed as previously described. Briefly, paraffin-embedded sections were dewaxed in xylene and hydrated using ethanol gradients. The slides were then boiled in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 min and soaked in 3% hydrogen peroxide (in methanol) for 10 min. After blocking with 5% goat serum for 1 h at room temperature, the slides were incubated with primary antibody overnight at 4° C. After washing, the slides were incubated with secondary antibody at room temperature for 0.5 h. The secondary antibody was coupled with HRP using ABC-HRP Kit (Vector laboratories, #PK-6101). The slides were developed using the DAB Kit (Vector laboratories, #SK-4100). For IF staining, the following primary antibodies were used: RFP (Rockland #600-401-379, IF 1:500); Ki67 (Invitrogen #14-5698-82, IF 1:500); HNF4a (Abcam #ab41898, IF 1:500), and the following secondary antibodies were used: Goat anti-rat IgG (H&L)

Alexa Fluor Plus 488 (Invitrogen #A-48262, IF 1:500); Donkey anti-rabbit IgG (H&L) Alexa Fluor 594 (Invitrogen #A-21207, IF 1:500); Goat anti-mouse IgG2a Alexa Fluor 647 (Invitrogen #A-21241, IF 1:500). IF was performed on paraffin embedded mouse liver sections using the same protocol as IHC except that secondary antibodies were substituted by Alexa Fluor conjugated antibodies. TUNEL staining was performed on paraffin embedded liver sections using In Situ Cell Death Detection Kit, Fluorescein (Roche #C755B40) according to the manufacturer's protocol. Sirius Red staining was performed on paraffin embedded liver sections using the Picro Sirius Red Staining Kit (Abcam #ab150681) according to the manufacturer's protocol. QuPath software was used to quantify TUNEL staining and IHC staining of Ki67. ImageJ was used to quantify Sirius Red staining.

Plasma and Liver Metabolic Assays

[0145] Blood was taken using heparinized tubes from the inferior vena cava immediately after sacrificing the mouse, and then transferred into 1.5 ml tubes and centrifuged at 2000 g for 15 min at 4° C. The supernatant after centrifugation (plasma) was analyzed for AST, ALT, cholesterol, and triglyceride (Manufacturer's Reference Numbers 8433815, 1655281, 1669829, and 1336544, respectively) using a fully automated OCD Vitros 350 dry chemistry analyzer following the protocols provided by the reagent kit manufacturer (Ortho Clinical Diagnostics, Raritan, NJ) at the UT Southwestern Metabolic Phenotyping Core. 100-150 mg of liver per mouse was weighed and used for lipid extraction and quantification at the UT Southwestern Metabolic Phenotyping Core. Briefly, flash frozen tissue samples were homogenized with 2:1 chloroform:methanol mixture (v/v) using a multiplexed automatic tissue disruptor (TissueLYser II, Qiagen, Germantown, MD). The organic extract was transferred to a 5 ml graduated flask and the total volume was brought up to 5 ml. Total cholesterol and triacylglycerol analyses were performed in triplicate using 100 µl and 25 µl of lipid extracts, respectively. Total cholesterol and triacylglycerol concentrations were determined by commercial enzymatic colorimetric assays following the protocols described by the manufacturer (INFINITY™ Cholesterol Liquid Stable Reagent #TR13421; INFINITY™ Triglycerides Liquid Stable Reagent #TR22421; Matrix PLUS™ Chemistry Reference Kit #NC9592194).

MOSAICS Reagent Construction

[0146] The MOSAICS plasmid uses the pX602 plasmid as a backbone. The sequence between the two AAV ITRs were removed using the NsiI and NotI restriction enzymes. The following fragments were cloned between the two AAV ITRs: the first SB100 binding IR, a U6 driven sgRNA scaffold, a CAG promoter driven SB100-P2A-Cre fusion cDNA with a beta-globin poly(A) signal, and the second SB100 binding IR. For library construction, mouse candidate genes for all of the in vivo screens were generated by using the mouse homologs of the human genes. A few genes were not included in the mouse gene lists due to the lack of a homolog or because they were known tumor suppressor genes. The individual sgRNA sequences corresponding to mouse candidate genes were extracted from the Brie library or obtained from the GUIDES server, and synthesized by CustomArray. Most genes had 5 distinct sgRNAs, 4 from Brie and 1 from GUIDES. A few genes had 4 targeting sgRNAs due to the overlap of sgRNA sequences from Brie and GUIDES. The library construction protocol was as well established in the field. Briefly, synthesized oligonucleotide libraries were amplified by PCR, purified using a PCR Purification Kit (Qiagen, #28104), and assembled in BsaI digested MOSAICS vector using DNA Assembly Kit (NEB, #E5520A). 1 µl of the assembled vector was then electroporated into 25 µl competent cells (Lucigen, #60052-2). After recovery in SOC medium for 1 h, bacteria were spread on a 245*245 mm LB agar plate and incubated at 37° C. overnight. The bacteria were then harvested for plasmid preparation using the HiSpeed Plasmid Maxi Kit (Qiagen, #12663). Each sgRNA maintained a >1000-fold representation during construction. For individual sgRNA cloning, forward and reverse primers were annealed and fused to BsaI digested MOSAICS plasmid using T4 ligase. See Table 5 for the primers associated with the sgRNAs and the sgRNA.

TABLE-US-00006 TABLE 5 sgRNA primers Sequence SEQ ID NO Tbx3-sgRNA-F CACCGGAGCACCTCACTTTAAACGG 116 Tbx3-sgRNA-R AAACCCGTTTAAAGTGAGGTGCTCC 117 Mouse TBX3-sgRNA1 GAGCACCTCACTTTAAACGG 318 Mouse TBX3-sgRNA1 CATCATGGATCAGTTAGTGG 319 Mouse TBX3-sgRNA1 AAAGAGAATGTATATACACC 320 Mouse TBX3-sgRNA1 TCCCAAGCGATCACGCAACG 321

AAV Production and Purification

[0147] AAV8 was produced using AAV-Pro 293T cells (Takara #632273) cultured in one or more 15 cm dishes. Cells were plated one day before transfection at 50% confluence, which would allow the cells to reach 80-90% confluence the next day. For transfection of one 15 cm dish, 10 μg MOSAICS vector, 10 μg pAAV2/8 (Addgene #112864) and 20 μg pAdDeltaF6 (Addgene #112867) plasmids were mixed with 1 ml Opti-MEM medium in one tube. In another tube, 160 μl PEI solution (1 mg/ml in water, pH7.0, powder from ChemCruz #sc-360988) was mixed with 1 ml Opti-MEM medium. The solutions from both tubes were then mixed and incubated for 10 min before adding to cell culture. 48 h after transfection, the cells were scraped off the dish and collected by centrifugation at 500 g for 10 min. The supernatant was disinfected and discarded, and the cell pellets were lysed in 1.5 ml/15 cm dish lysis buffer (PBS supplemented with NaCl powder to final concentration of 200 mM, and with CHAPS powder to final concentration of 0.5% (w/v)). The cell suspension was put on ice for 10 min with intermittent vortexing, and then centrifuged at 20,000 g for 10 min at 4° C. The supernatant containing the AAV was collected. To set up the gravity column for AAV purification, 0.5 ml of AAV8-binding slurry beads (ThermoFisher #A30789), enough to purify AAV from three 15 cm dishes, was loaded into an empty column (Bio-Rad #731-1550). After the beads were tightly packed at the bottom, they were washed with 5 ml of wash buffer (PBS supplemented with NaCl powder to a final concentration of 500 mM). The supernatant containing AAV was then loaded onto the column. After all of the supernatant flowed through, the beads were washed with 10 ml wash buffer twice. The AAV was then eluted with 3 ml elution buffer (100 mM glycine, 500 mM NaCl in water, pH 2.5) and the eluate was immediately neutralized with 0.12 ml 1M Tris-HCl (pH 7.5-8.0). The AAV was concentrated by centrifugation at 2000 g for 3-5 min at 4° C. using an 100 k Amicon Ultra Centrifugal Filter Unit (Millipore #UFC810024). After centrifugation, the volume of AAV should be equal to or less than 0.5 ml. The concentrated AAV was diluted with 4-5 ml AAV dialysis buffer (PBS supplemented with powders to final concentrations of 212 mM NaCl and 5% sorbitol (w/v)) and centrifuged at 2000 g for 3-5 min at 4° C. The dilution and centrifugation processes were repeated 3 times. The final concentrated AAV was transferred into a 1.5 ml tube and centrifuged at 20,000 g for 5 min to remove debris. The supernatant was aliquoted, flash frozen using liquid nitrogen, and stored at −80° C.

Genomic DNA Extraction, sgRNA Amplification, and Amplicon Library Construction

[0148] To extract genomic DNA containing the integrated sgRNA, the entire liver (except a small piece used for sectioning and H&E staining) was minced into about 1 mm.sup.3 pieces using a blade and weighed. Small nodules observed in some epigenetic factor screening livers given WD (<=3 nodules per liver in 5 out of 8 livers) were excluded from samples being processed for genomic DNA extraction. Minced liver in two volumes (w/v) of homogenizing buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 8) was transferred into a glass Wheaton Dounce Tissue Grinder and stroked 50 times or until no bulk tissues were seen. After homogenizing, 200 μl chow fed liver lysate or 300 μl WD fed liver lysate was transferred to a 15 ml tube for genomic DNA extraction using the Blood & Cell Culture DNA Midi Kit (Qiagen #13343) according to the manufacturer's protocol. The remaining lysates were frozen in −80° C. as backup samples. Briefly, 10 ml Buffer G2 from the kit, 100 pl Proteinase K (Roche #03115828001, or Proteinase K from the Qiagen kit) and 100 pl RNase A (Invitrogen #12091-021) were added to the 15 ml tube containing the lysate and digested in a 50° C. water bath overnight. The next day,

the tubes were centrifuged at 4000 g for 10 min and the lipid layer on the top was discarded. The remaining supernatant was loaded on the column, washed, and genomic DNA elution/precipitation were performed according to the manufacturer's protocol. The precipitated DNA was resuspended in 100 pl 10 mM Tris (pH 8.0) and shaken on a 55° C. shaker for 2 h to help it dissolve. For amplicon library preparation, 5 pg genomic DNA, 5 pl general forward primer mix (5 pM), 5 pl barcode specific reverse primer (5 pM), 1 pl Q5 DNA polymerase, 10 pl Q5 buffer, 10 pl HighGC buffer, 1 pl dNTP, and water was mixed for a 50 pl PCR reaction, and two reactions were made for each genomic DNA sample. The PCR cycle was 95° C. 3 min-(95° C. 30 s-60° C. 30 s-72° C. 20 s)*n−72° C. 2 min. The PCR cycle number was pre-optimized using the same PCR reactions with a smaller volume. The cycle numbers that gave a weak but sharp band on the DNA gel were used. In the final PCR reaction, 23 cycles were used for preparing the NASH gene, transcription factor, and epigenetic factor screens, and 30 cycles were used for preparing the guide mini-pool validation screen. After PCR, the two tubes of reactions with the same genomic DNA template were combined (total 100 pl) and 70 pl was resolved on a DNA gel. The 250 bp band corresponding to the amplicon was cut and purified using the QIAquick Gel Extraction Kit (Qiagen #28704). The DNA concentration was determined using Qubit kit (Invitrogen #Q32853) and high-throughput sequencing was performed using an Illumina NextSeq500 system at the CRI at UT Southwestern Sequencing Facility.

Bioinformatic Analysis of MOSAICS Screening Results

[0149] The reads from the sequencing of amplicon libraries described above were trimmed with cutadapt (version 1.9.1) to remove the excessive adaptor sequences so that only the sgRNA sequences were retained. The 5′ sequences were trimmed with the options —O 32-discard-untrimmed-g CTTTATATATCTTGTGGAAAGGACGAAACACCG. The 3′ sequences were trimmed with the options —O 12-a GTTTTAGAGCTAGAAATAGCA. The abundance of each sgRNA was calculated with the count function in MAGeCK (version 0.5.6) with the default option. The trimmed fastq files were assigned to chow-fed and WD-fed groups and uploaded together with library files containing sgRNA sequences and targeted gene names to a server preloaded with MAGeCK. The enrichment of each sgRNA was calculated with the test function in MAGeCK.

RNA-Seq Library Preparation and Transcriptome Analysis of Mouse Fatty Livers

[0150] Total liver RNA was extracted from 4 sgGFP, 2 sgLacZ, 7 Tbx3, KO livers using TRIzol reagent (Invitrogen #15596026) followed by purification using the RNeasy Mini kit (Qiagen #74014). Briefly, a liver fragment with a volume of about 3*3*3 mm.sup.3 from each sample was homogenized in 1 ml Trizol, followed by adding 200 pl chloroform and vortexed. After centrifugation at 20,000 g for 10 min at 4° C., 350 pl supernatant from each sample was transferred to a new tube and mixed with equal volume of 75% EtOH, and then loaded on an RNeasy column. The following wash steps using RW1 and RPE buffers and RNA precipitation step were performed according to the manufacturer's protocol. RNA-seq libraries were prepared with the SMARTer Stranded Total RNA Sample Prep Kit—HI Mammalian (Takara #634875). 75 bp single-end sequencing was performed using an Illumina NextSeq500 system at the CRI at UT Southwestern Sequencing Facility. Alignment, quantification, and differential expression analysis were performed using the QBRC_BulkRnaSeqDE pipeline (https://github.com/QBRC/QBRC_BulkRnaSeqDE). Briefly, the alignment of reads to the mouse reference genome (mm10) was done using (v2.7.2b) FeatureCounts (v1.6.4) was then used for gene count quantification. Differential expression analysis was performed using the R package DEseq2 (v1.26). Cutoff values of absolute fold change greater than 2 and FDR<0.05 were used to select for differentially expressed genes between sample group comparisons. Finally, GSEA was carried out with the R package fgsea (v1.14.0) using the ‘KEGG’ and ‘Hallmark’ libraries from MsigDB.

Transcriptomic Analysis of Human NASH Livers

[0151] Dataset 1 (NAFLD/NASH cohort): The RNA-Seq transcriptome profiles of biopsied liver tissues (GEO: GSE130970) were downloaded and analyzed 72 NAFLD/NASH patients with a

range of disease severities (NASH activity scores of 1 to 6) and relevant histological features, i.e., steatosis, inflammation, fibrosis, and hepatocyte ballooning. The raw sequence reads were aligned to the GENCODE human reference genome (GRCh37, p13) using the STAR aligner (ver 2.6.1b), and gene-level count data were generated by the feature Counts function in the Subread package (ver 1.6.1), and the GENCODE genome annotation (GRCh37, v19). The count data were normalized using “Relative Log Expression” normalization (RLE) implemented in the DESeq2 package Dataset 2 (HCV cirrhosis cohort): The microarray gene expression profile of formalin-fixed needle biopsy specimens from the livers of 216 patients with hepatitis C-related early-stage (Child-Pugh class A) cirrhosis (GEO: GSE156540) was analyzed. This cohort was prospectively followed for a median of 10 years at an Italian center with relevant time event outcomes collected, including child, death, HCC and decomposition. Male and female patients were included in these studies.

Curating Transcription and Epigenetic Factors with Putative Pathogenic Activity in NAFLD/NASH

[0152] Gene expression of transcription factors do not directly reflect their functional activities, due to the low correlation between gene expression and protein abundance as well as the co-regulation with co-factors. Transcription factor activities can be estimated using enrichment of their downstream targets. However, traditional methods such as Gene Set Enrichment Analysis (GSEA) overestimate the number of significant transcription factors, largely confounded by the large number of overlaps among putative target genes of different TFs. Therefore, global modeling of putative transcription factors was performed with gene expression data from NAFLD/NASH (GEO: GSE130970) and HCV cirrhosis patients (GEO: GSE15654) to directly infer the transcription factor activities from their downstream targets with adjustment for overlapping targets. A similar linear regression-based model was previously proposed to predict transcription factor regulatory activities and motifs from yeast gene expression data. The method regresses the fold-change of a gene on its putative regulatory transcription factor(s). The coefficient (Z score) of a transcription factor, estimated using genome-wide fold changes and predicted targets of all transcription factors, represents the regulatory activity change of the transcription factor across all the liver patients.

[0153] The regression model is defined as following:

[00001] $f_q = \sum_i a_i T_{ig} + \sum_j \sum_i M_{jg} + c$

where f.sub.g is the fold change of g-th gene between two conditions; T.sub.ig is the number of binding sites of i-th TF on the promoter of the g-th gene; M.sub.ig is the number of binding sites of the j-th microRNA on the 3′ UTR of the g-th gene; and α.sub.i, β.sub.j and c (a constant) can be inferred based on the values of f.sub.g T.sub.ig and M.sub.jg for all the genes in the RNA-seq data. The Z scores of coefficients α.sub.i and β.sub.j represent the activity changes of the i-th TF and j-th microRNA. Global transcription factor binding sites represented by 190 position-weighted matrices (PWMs) covering 500 mammalian TFs were based on the union of JASPAR, TRANSFAC and additional motifs from chromatin immunoprecipitation with DNA microarray and ChIP-seq data. The initial regression analysis was done using ISMARA before further integrative analysis with patient clinical histological features and time event outcome; sample-specific transcription factor activity was estimated by the same regression model, where the fold changes were calculated between a single sample and all the samples combined together.

[0154] In the NAFLD/NASH cohort, the sample-level activities for each TF were associated with the four histological features, including fibrosis, inflammation, ballooning, and steatosis based on Pearson correlation. In the HCV cirrhosis cohort, the activities for each TF were used to perform outcome analysis on four-time events, including child, death, HCC and decomposition using cox proportional regression model. The p-values were calculated for both analyses respectively, followed by the calculation of False Discovery Rate (FDR) for multiple testing correction.

[0155] Similarly, to screen the putative pathogenic epigenetic regulators, the Pearson correlation was calculated between the gene expression of epigenetic regulators and the four histological

features (fibrosis, inflammation, ballooning, and steatosis) in the NAFLD/NASH cohort, and used gene expression of known epigenetic regulators as independent variables to perform the cox proportional regression on four time events (Child-Pugh, death, HCC, decompensation) in the HCV cirrhosis cohort. The p-values were calculated for both analyses respectively, followed by the calculation of FDR for multiple testing correction.

Quantification and Statistical Analysis

[0156] The data in most panels reflect multiple experiments performed on different days using mice derived from different litters. Variation in all panels is indicated using standard deviation presented as mean±SD. Two-tailed unpaired Student's t-tests were used to test the significance of differences between two groups. Statistical significance is displayed as ns (not significant, or $p>=0.05$), *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$) unless specified otherwise. Image analysis for quantification was blinded.

Claims

1. A composition comprising a nucleic acid that downregulates expression of TBX3 (T-box transcription factor 3) or a variant thereof.

2. The composition of claim 1, wherein the nucleic acid that downregulates expression of TBX3 comprises a siRNA, a cluster regularly interspaced short palindromic repeats (CRISPR) related nucleic acid, a single guide RNA (sgRNA), a CRISPR-RNA (crRNA), or a trans-activating crRNA (tracrRNA).

3. The composition of claim 2 wherein the nucleic acid is a small interfering RNA (siRNA) molecule.

4. A composition comprising a plasmid or a viral vector, wherein the plasmid or the viral vector comprises a nucleic acid encoding the siRNA molecule of claim 3.

5. The composition of any one of claim 3 or 4, wherein the siRNA molecule comprises a nucleotide sequence that is 2 to 30 nucleotides in length and is at least 80% homologous to at least 2 to 30 contiguous nucleotides of a human TBX3 cDNA sequence.

6. The composition of claim 5, wherein the human TBX3 cDNA sequence comprises SEQ ID NO: 1.

7. The composition of any one of claim 3 or 4, wherein the siRNA molecule targets the open reading frame or the 5′ or 3′ UTRs of the TBX3 gene.

8. The composition of any one of claims 3-7, wherein the siRNA molecule comprises at least one sense sequence, at least one antisense sequence, or at least one sense sequence and at least one antisense sequence.

9. The composition of any one of claims 3-8, wherein the siRNA molecule comprises a nucleotide sequence SEQ ID NOs: 2-115 or any combination thereof.

10. The composition of claim 9, wherein the at least one sense sequence comprises SEQ ID NOs: 2-58.

11. The composition of claim 9, wherein the at least one antisense sequence comprises SEQ ID NOs: 59-115.

12. The composition of claim 2, wherein the nucleic acid that downregulates expression of TBX3 is a sgRNA molecule optionally selected from a group consisting of SEQ ID NO: 118-217.

13. A composition comprising a plasmid or a viral vector, wherein the plasmid or viral vector comprises a first nucleic acid encoding the sgRNA molecule of claim 12 and optionally a second nucleic acid encoding an RNA guided nuclease.

14. The composition of claim 13, wherein the RNA guided nuclease is a Cas endonuclease.

15. The composition of any one of claims 3-11 wherein the siRNA molecule specifically downregulates gene expression of at least one variant of TBX3.

16. The composition of any one of claims 12-14, wherein the sgRNA molecule specifically

downregulates gene expression of at least one variant of TBX3.
17. The composition of any one of claim 15 or 16, wherein the at least one variant of TBX3 is associated with a liver disease.
18. The composition of claim 17, wherein the liver disease comprises fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer, or any combination thereof.
19. The composition of any one of claims 1-3 wherein the nucleic acid molecule is conjugated to least one targeting ligand.
20. The composition of claim 19, wherein the at least one targeting ligand comprises a liver targeting ligand.
21. The composition of claim 20, wherein the liver targeting ligand comprises at least one N-acetylgalactosamine (GalNAc) conjugate.
22. The composition of claim 21, wherein the nucleic acid molecule is conjugated to about one to about three GalNAc conjugates.
23. The composition of any one of claims 1-3 or 12, wherein the nucleic acid that specifically downregulates expression of TBX3 comprises at least one chemical modification.
24. The composition of claim 23, wherein the nucleic acid comprises a modification at least one ribosugar moiety of its nucleotide sequence.
25. The composition of claim 24, wherein at least one ribosugar moiety is modified with 2 2′-O-methyl (2′OMe), 2′-deoxy-2′-fluoro (2′F), 2′-deoxy, 5-C-methyl, 2′-O-(2-methoxyethyl) (MOE), 4′-thio, 2′-amino, 2′-C-allyl, or any combination thereof.
26. The composition of either claim 24 or claim 25, wherein less than about 10% to about 70% of ribosugar moieties of the total nucleotide sequence is modified.
27. A pharmaceutical composition comprising any one of the compositions of claims 1-26 and at least one pharmaceutically acceptable carrier.
28. The pharmaceutical composition of claim 27, further comprising a nanoparticle.
29. The pharmaceutical composition of either claim 27 or claim 28, further comprising a lipid.
30. A method of for treating a subject in need thereof, the method comprising administrating a therapeutically effective amount of the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29 to the subject in need thereof.
31. The method of claim 30, wherein the subject in need thereof, is a human subject having or suspected of having a liver disease.
32. The method of claim 31, wherein the liver disease comprises fatty liver disease (FLD), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), end stage liver disease (cirrhosis) from any etiology, liver cancer, or any combination thereof.
33. The method of any one of claims 30-32, wherein the method of administering comprises parenteral administration.
34. The method of any one of claims 30-33, wherein administration of a therapeutically effective amount of the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29 increases life expectancy of the subject compared to an untreated subject with identical disease condition and predicted outcome.
35. The method of any one of claims 30-33, wherein administration of a therapeutically effective amount of the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29 improves liver function of the subject compared to an untreated subject with identical disease condition and predicted outcome.
36. The method of any one of claims 30-33, wherein administration of a therapeutically effective amount of the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29 attenuates liver fibrosis in the subject compared to an untreated subject with

identical disease condition and predicted outcome.

37. The method of any one of claims 30-33, wherein administration of a therapeutically effective amount of the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29 prevents additional liver fibrosis in the subject compared to an untreated subject with identical disease condition and predicted outcome.

38. A kit comprising: a. a container holding the composition of any one of claims 1-26 or the pharmaceutical composition of any one of claims 27-29; b. a pharmaceutical administrative means; and c. an instruction.