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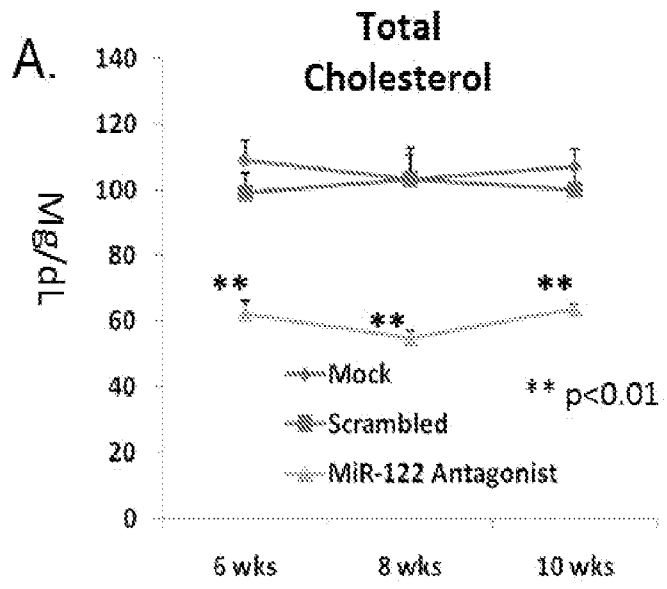

FIGURE 4

(57) Abstract: The invention in some aspects relates to methods and compositions for assessing the effectiveness of miRNA inhibitors. In other aspects of the invention, methods and compositions for treating cholesterol related disorders are provided. In one aspect of the invention, miRNA inhibitors against miR-122 and rAAV-based compositions comprising the same are provided.

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AAV-BASED TREATMENT OF CHOLESTEROL-RELATED DISORDERS

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119 of U.S. provisional application USSN 61/327,383, filed April 23, 2010, and entitled "AAV-based treatment of cholesterol-related disorders," the entire contents of which are incorporated herein by reference.

GOVERNMENT SUPPORT

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FIELD OF THE INVENTION

The invention in some aspects relates to methods and compositions for assessing the effectiveness of miRNA inhibitors. In other aspects of the invention, methods and compositions for treating cholesterol related disorders are provided.

BACKGROUND OF INVENTION

Dyslipidemia is associated with defects in cholesterol metabolism and represents a major risk factor for cardiovascular disease, the most common cause of morbidity and mortality in the US. One common inherited form of dyslipidemia is the metabolic defect in low density lipoproteins (LDL) [familial hypercholesterolemia (FH)] caused by genetic mutations in the LDL receptor (*LDLR*) gene. MicroRNAs (miRNAs) are small regulatory RNAs that are important in development and progression of disease. It is understood that certain microRNAs have a role cholesterol metabolism. A highly abundant miRNA in the liver, miR-122, which does not directly target LDLR mRNA, regulates cholesterol metabolism by an unknown mechanism. A locked nucleic acid based oligonucleotide inhibitor of miR-122 has been shown to reduce total plasma cholesterol levels in a dose dependent manner (See, *e.g.*, Elmen J, et al. Nature, 2008, 452: 896-900.) However, such oligonucleotide based inhibitors require doses impractical for a therapeutic agent. Furthermore, since the oligonucleotides are administered in finite quantities, repeated administration is required to maintain a long term inhibitory effects, which are necessary for

many cholesterol-related disorders, like FH. Notwithstanding the link between miRNAs and cholesterol, and prospects of effective therapeutic agents that treat cholesterol-related disorders by modulating miRNA function, the development of effective and safe approaches for miRNA inhibition in the treatment of cholesterol related disorders has been a significant scientific and therapeutic challenge (See, *e.g.*, Czech, MP. N Engl. J. Med. 354; 11 pg. 1144-1145. (2006).)

SUMMARY OF INVENTION

Aspects of the invention are based on molecular sensing systems that enable the assessment and characterization of miRNA inhibitor function and thereby facilitate the discovery of miRNA inhibitors that are useful for treating and studying disease, *e.g.*, cholesterol-related disorders. According to some aspects of the invention, miRNA inhibitors are identified herein that are useful for treating cholesterol-related disorders. In some embodiments, rAAV-based miRNA inhibitor compositions are used to effect sustained, tissue specific miRNA inhibition in a subject. In some aspects, a rAAV of the invention harbors at least one transgene that expresses a miRNA inhibitor that inhibits the function, processing and/or expression of miR-122 in the subject. An exemplary miRNA inhibitor of the invention has a sequence as set forth in SEQ ID NO: 1.

According to some aspects of the invention, methods are provided for treating a high cholesterol-related disorder in a subject. In some embodiments, the methods involve administering to a subject an effective amount of a rAAV that harbors at least one transgene that expresses a miRNA inhibitor that inhibits the expression of miR-122 in the subject. In some embodiments, the miRNA inhibitor comprises an miR-122 binding site. In some embodiments, the miR-122 binding site is flanked by two stem sequences. In some embodiments, the miR-122 binding site comprises a non-binding, central portion that is not complementary with miR-122, flanked by two portions that are complementary with miR-122. In some embodiments, the miRNA inhibitor comprises a first miR-122 binding site and a second miR-122 binding site, each binding site flanked by two stem sequences, wherein a first stem sequence flanks the first miR-122 binding site at its 5'-end, a second stem sequence flanks the first miR-122 binding site at its 3'-end and the second miR-122 binding site at its 5'-end, and a third stem sequence flanks the second miR-122 binding site at its 3'-end. In some embodiments, each of the two miR-122 inhibitor binding sites comprises a non-binding, central portion that is not complementary with miR-122. In some embodiments, the non-

binding, central portion of the first miR-122 binding site is at least partially complementary with the non-binding, central portion of the second miR-122 binding site. In some embodiments, the non-binding, central portion of the first miR-122 binding site is complementary with the non-binding, central portion of the second miR-122 binding site at 1 to 5 nucleotides. In some embodiments, the non-binding, central portion of the first miR-122 binding site is complementary with the non-binding, central portion of the second miR-122 binding site at 3 nucleotides. In some embodiments, the non-binding, central portion of the first miR-122 binding site has a length in a range of 1 to 10 nucleotides. In some embodiments, the non-binding, central portion of the first miR-122 binding site has a length in a range of 3 to 5 nucleotides. In some embodiments, the non-binding, central portion of the first miR-122 binding site has a length in a range of 4 nucleotides. In some embodiments, the non-binding, central portion of the second miR-122 binding site has a length in a range of 1 to 10 nucleotides. In some embodiments, the non-binding, central portion of the second miR-122 binding site has a length in a range of 3 to 5 nucleotides. In some embodiments, the non-binding, central portion of the second miR-122 binding site has a length in a range of 4 nucleotides. In some embodiments, the first miR-122 binding and the second miR-122 binding site are complementary at a sequence of 2 to 10 nucleotides in length. In some embodiments, the first miR-122 binding and the second miR-122 binding site are complementary at a sequence of 4 nucleotides in length. In some embodiments, the miRNA inhibitor comprises two or more miR-122 binding sites. In certain embodiments, the miRNA inhibitor has a sequence as set forth in SEQ ID NO: 1.

In certain embodiments, the rAAV has a capsid of the AAV9 serotype, which has a sequence as set forth in SEQ ID NO: 3. In some embodiments, the rAAV has a capsid that is a variant of the capsid of the AAV9 serotype. In certain embodiments, the rAAV has a capsid of the AAV9 serotype variant, Csp-3, which has a sequence as set forth in SEQ ID NO: 4. In some embodiments, the rAAV targets liver tissue. In some embodiments, the rAAV transduces hepatocytes. In certain embodiments, the effective amount of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, or 10^{14} genome copies per kg. In certain embodiments, the effective amount of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, or 10^{15} genome copies per subject.

In some embodiments, administering is performed intravenously. In some embodiments, administering is performed by injection into the hepatic portal vein. In some embodiments, the subject is a mouse, a rat, a rabbit, a dog, a cat, a sheep, a pig, or a non-human primate. In some embodiments, the subject is a human. In some embodiments, the

subject is an animal model of a high cholesterol-related disorder. In some embodiments, the high cholesterol-related disorder is Type I, Type IIa, Type IIb, Type III, Type IV, or Type V Hyperlipoproteinemia. In some embodiments, the high cholesterol-related disorder is associated with diabetes mellitus, metabolic syndrome, kidney disease (nephrotic syndrome), hypothyroidism, Cushing's syndrome, anorexia nervosa, sleep deprivation, Zieve's syndrome, antiretroviral drugs, diet, high body weight, or low physical activity. In some embodiments, the subject is a human and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 200 mg/dl. In some embodiments, the subject is a mouse and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 100 mg/dl. In some embodiments, the subject is a rat and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 70 mg/dl.

According to some aspects of the invention a nucleic acid vector is provided for assessing the function of a miRNA inhibitor. In some embodiments, the nucleic acid vectors comprise: (a) a first promoter operably linked with a transgene that comprises: (i.) a protein coding region, and (ii.) at least one binding site of a test miRNA; and (b) a second promoter operably linked with a miRNA inhibitor coding region, wherein the miRNA inhibitor hybridizes with the test miRNA. In some embodiments, the first promoter is a RNA Polymerase II promoter. In some embodiments, the second promoter is a RNA Polymerase III promoter. In some embodiments, the nucleic acid vector further comprises a first untranslated region between the first promoter and at least a portion of the protein coding region, wherein the second promoter and the miRNA inhibitor coding region are positioned within the first untranslated region. In some embodiments, the first untranslated region is positioned at the 5' end of the complete protein coding region. In some embodiments, the first untranslated region is positioned within an intron of the protein coding region. In some embodiments, the transgene further comprises a second untranslated region, wherein the at least one binding site of the test miRNA is in the second untranslated region. In some embodiments, the second untranslated region is positioned at the 3' end of the complete protein coding region. In some embodiments, the nucleic acid vector further comprises a pair of inverted terminal repeats that flank the first promoter and the transgene. In some embodiments, the pair of inverted terminal repeats further flank the second promoter and the miRNA inhibitor coding region. In some embodiments, the protein coding region encodes a reporter protein selected from: a

fluorescent protein, luciferase, β-galactosidase, secreted alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase (CAT), and β-lactamase.

In some aspects of the invention, a molecule sensing system is provided. In some embodiments, the molecular sensing system comprises a nucleic acid vector for assessing the function of a miRNA inhibitor. In some embodiments, the nucleic acid vector of the molecular sensing system comprises a promoter operably linked with a transgene that is regulated by a test miRNA and a promoter operably linked with a miRNA inhibitor coding region.

According to some aspects of the invention, methods are provided for assessing the effectiveness of a miRNA inhibitor. In some embodiments, the methods comprise (a) transfecting a cell with any of the foregoing nucleic acid vectors, wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; and (b) determining the level of expression of the protein encoded by the protein coding region in the cell, wherein the level of expression of the protein is indicative of the effectiveness of the miRNA inhibitor. In some embodiments, the methods further comprise contacting the cell with the test miRNA. In some embodiments, the cell expresses the test miRNA. In some embodiments, the methods comprise (a) transfecting a first cell with any one of the foregoing nucleic acid vectors, wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; (b) transfecting a second cell with the nucleic acid vector, wherein levels of the test miRNA are lower in the second cell compared with the first cell; and (c) comparing the level of expression of the protein encoded by the protein coding region in the first cell with the level of expression of the protein encoded by the protein coding region in the second cell, wherein the results of the comparison in (c) are indicative of the effectiveness of the miRNA inhibitor. In some embodiments, the methods comprise (a) transfecting a cell with any one of the foregoing nucleic acid vectors, wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; (b) determining a first level of expression of the protein encoded by the protein coding region in the cell; (c) contacting the cell with the test miRNA; (d) determining a second level of expression of the protein encoded by the protein coding region in the cell; and (e) comparing the first level of expression of the protein with the second level of expression, wherein the results of the comparison in (e) are indicative of the effectiveness of the miRNA inhibitor. In some embodiments, the methods comprise (a) transfecting a cell with any one of the foregoing nucleic acid vectors, wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; (b) determining a first level of expression of the protein encoded by the

protein coding region in the cell; and (c) comparing the first level of expression of the protein with a control level of expression, wherein the results of the comparison in (c) are indicative of the effectiveness of the miRNA inhibitor.

In some aspects of the invention, kits are provided for assessing the function of a miRNA inhibitor. In some embodiments, the kits comprise a container housing any of the foregoing nucleic acid vectors. In some embodiments, the kits comprise a container housing a component of a molecular sensing system.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 depicts a molecular sensing system for evaluating miRNA inhibitor function.

Figure 2A depicts results from a molecular sensing system assay showing that TuD miR-122 Inhibitor expressed from a polymerase III promoter is highly effective at derepressing reporter gene expression in 293 cells compared with other putative miRNA inhibitors.

Figure 2B depicts results from a molecular sensing system assay showing that TuD miR-122 Inhibitor expressed from a polymerase III promoter completely restored reporter gene expression in Huh-7 cells from a nucleic acid vector having a single miR122 and substantially derepressed reporter gene expression in Huh-7 cells from a nucleic acid vector having three miR122 binding sites compared with other putative miRNA inhibitors.

Figure 2C depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively knocks down mature free miR-122 in the liver of mice infected with a rAAV9 containing the vector.

Figure 3A depicts the sequence and structural features of the TuD miR-122 Inhibitor (SEQ ID NO: 1).

Figure 3B depicts the predicted secondary structure of the TuD miR-122 Inhibitor (SEQ ID NO: 1). Structure predicted using MFOLD (mobyle.pasteur.fr/cgi-bin/portal.py?form=mfold)

Figure 3C depicts the sequence and structural features of the TuD Let-7 Inhibitor (SEQ ID NO: 2).

Figure 4A depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced total serum cholesterol levels for up to 10 weeks in mice infected with a rAAV9 containing the vector and fed a normal chow diet.

Figure 4B depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced serum HDL levels for up to 10 weeks in mice infected with a rAAV9 containing the vector and fed a normal chow diet.

Figure 4C depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced serum LDL levels for up to 2 weeks in mice infected with a rAAV9 containing the vector and fed a normal chow diet.

Figure 5A depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced total serum cholesterol levels for up to 10 weeks in $LDLR^{-/-}$ $Apobec1^{-/-}$ mice (a model Familial hypercholesterolemia) infected with a rAAV9 containing the vector.

Figure 5B depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced serum HDL levels for up to 2 weeks in $LDLR^{-/-}$ $Apobec1^{-/-}$ mice infected with a rAAV9 containing the vector.

Figure 5C depicts results from an *in vivo* assay showing that rAAV vector expressing a TuD miR-122 Inhibitor effectively reduced serum LDL levels for up to 2 weeks in $LDLR^{-/-}$ $Apobec1^{-/-}$ mice for infected with a rAAV9 containing the vector.

Figure 6 Structure of a Tough Decoy miR-122 (TuD) RNA (SEQ ID NO: 5). TuD RNAs contain two single-stranded miRNA binding sites flanked by double-stranded stems intended to enhance stability and promote nuclear export.

Figure 7 Comparison of miR-122 inhibitor strategies in cultured cells. (**a**) miRNA inhibitor constructs. (**b**) Pairing of antagonists to miR-122. Upper sequence in all three panels: miR122 fragment (SEQ ID NO: 6). Sponge: SEQ ID NO: 7. TuD: SEQ ID NO: 8. ZIP: SEQ ID NO: 9. (**c**) Plasmid harboring *nLacZ* reporter gene with one or three sites complementary to miR-122 was co-transfected with pTBG Fluc and either control plasmid, anti-miR-122 sponge plasmid or U6-driven anti-miR-122 TuD plasmid. The cells were stained for LacZ

expression 48 h after transfection, and blue cells were counted. Data are reported relative to a control reporter plasmid lacking miR-122-binding sites. (**d**) Reporter plasmid expressing *nLacZ* mRNA containing 3 miR-122-binding sites was co-transfected into HuH-7 cells with a U6-driven sponge-, miRZip- or TuD-expressing plasmid. The empty plasmid served as the control. (**e**) HEK 293 cells were transfected with a *nLacZ* reporter plasmid containing three fully complementary miR-122-binding sites, together with the constructs expressing anti-*let-7* or anti-miR-122 TuD transcribed from a U6 promoter or anti-miR122 sponge or anti-*let-7* sponge transcribed from an SV40 promoter, as well as different amounts of a plasmid producing pri-miR-122 RNA. Forty-eight hours later, the cells the percentages of nLacZ positive cells, relative to the control (nLacZ without miR-122binding sites), were determined (**c, d, and e**). (**f**) HuH-7 cells were transfected with reporter plasmid expressing control luciferase, luciferase bearing seven miR-122 binding sites, or seven mutant sites, as well as control plasmid or plasmid expressing anti-miR-122-, anti-*let-7* or scrambled TuD RNA. Twenty-four hours later, crude cell lysates were prepared and luciferase activity assayed. The data are presented as mean ± standard deviation for firefly luciferase activity normalized to *Renilla* luciferase activity.

Figure 8 Evaluation of *let-7* antagonist constructs in HeLa cells. (**a**,**b**) Total RNA and protein were prepared from HeLa cells transfected with the constructs expressing either anti-miR-122 or anti-*let-7* TuD, anti-*let-7* sponge or control plasmid. The relative levels of *Dicer* mRNA was measured by qRT-PCR (**a**) and of Dicer protein by Western blotting (**b**). The figure reports mean ± standard deviation.

Figure 9 Western blot analysis of HeLa cells transfected with the constructs expressing either anti-miR-122 or anti-*let-7* TuD, anti-*let-7* sponge or plasmid control. Three biological replicates are shown; Fig. 2c reports the quantification of these data.

Figure 10 Real-time monitoring of endogenous miRNA activity using miRNA sensor system. (**a**) Schematic presentation of *Gaussia* luciferase-(Gluc) expressing vectors. CB, chicken β actin promoter with CMV enhancer. AAV vector plasmids were transfected into HuH-7 (**b**) or HeLa cells (**c**)**.** Forty-eight hours later, Gluc activity was measured. (**d**, **e**) C57BL/6 mice were administered 1×10^{12} genome copies of scAAV9 per animal by tail vein injection**.** Blood was collected at the indicated times and assayed for Gluc activity. Gluc expression is reported as mean ± standard deviation, relative to samples from mice injected with a scAAV9 vector expressing Gluc but lacking both the TuD expression cassette and the 3′ UTR miRNA-binding sites. Each group had four mice.

Figure 11 Analysis of miRNA expression in liver from mice administered scAAV9 expressing anti-miRNA TuD. C57BL/6 mice were injected via tail vein with 1×10^{12} genome copies of control, anti-miR-122 or anti-*let-7* TuD expressing vectors. The animals were sacrificed four weeks later, and total liver RNA was prepared for qRT-PCR (**a**) and Northern blot (**b**) analyses of *let-7*, miR-122, miR-26a, miR-22 and U6. Data are presented as mean ± standard deviation. U6 RNA provided a loading control. (**c**, **d**) High throughput sequencing of total liver small RNA was used to determine the length distribution and abundance of genome-matching miR-122 (**c**) or prefix-matching miR-122 (**d**) four weeks after scAAV injection. The most abundant non-genome matching nucleotides added to the 3′ end of miR-122 fragments are indicated in the grey boxes. (**e**) Eight *let-7* isoforms are expressed in mouse liver. Nucleotide differences among the *let-7* isoforms are indicated in black and their pairing to anti-*let-7* TuD RNA is shown. The "seed" sequence, an important feature for miRNA-directed target RNA recognition, is underlined. Let-7a: SEQ ID NO: 10, Let-7b: SEQ ID NO: 11, Let-7c: SEQ ID NO: 12, Let-7d: SEQ ID NO: 13, Let-7e: SEQ ID NO: 14, Let-7f: SEQ ID NO: 15, Let-7g: SEQ ID NO: 16, Let-7i: SEQ ID NO: 17, TuD: SEQ ID NO: 18. (**f**) The anti-*let-7* TuD decreased the abundance of full-length *let-7* and increased the number of prefix-matching *let-7* sequence reads, relative to the control. Isoforms that decreased more than four-fold in genome-matching reads and increase in prefix-matching reads are shown in black.

Figure 12 Northern blot analysis of *let-7*, miR-122, miR-26a, miR-22 and U6 small nucleolar RNA (U6 snoRNA) in total RNA from liver of C57BL/6 mice injected with 1×10^{12} genome copies of scAAV9CBGluc (mock), scAAV9CBGlucTuDmiR-122 (anti-miR-122 TuD) or scAAV9CBGlucTuD*let-7* (anti-*let-7* TuD) via tail vein injection. The animals were sacrificed 4 weeks after injection and total liver RNA was prepared. Three biological replicates are shown and analyzed to generate Fig. 4b.

Figure 13 Length distribution and abundance of genome-matching or prefix-matching *let-7* isoform sequence reads in liver of mice 4 weeks after injection of scAAV9CBGluc (mock), scAAV9CBGlucTuD *let-7* (anti-*let-7* TuD). The most abundant non-templated nucleotides added to the 3′ end of the miR-122 prefixes are indicated in the grey boxes. Let-7a: SEQ ID NO: 10, Let-7b: SEQ ID NO: 11, Let-7c: SEQ ID NO: 12, Let-7d: SEQ ID NO: 13, Let-7e: SEQ ID NO: 14, Let-7f: SEQ ID NO: 15, Let-7g: SEQ ID NO: 16, Let-7i: SEQ ID NO: 17, TuD: SEQ ID NO: 18.

Figure 14 Abundance of miRNAs in liver of mice 4 weeks after injection of

scAAV9CBGluc (mock), scAAV9CBGlucTuD *let-7* (anti-*let-7* TuD) or scAAV9CBGlucTuDmiR-122 (anti-miR-122 TuD). Pearson correlation analysis was performed using GraphPad Prism V5.0b (GraphPad Software, Inc.). The correlation coefficient (*r*) and *p*-value are indicated. miRNAs targeted by TuDs are red.

Figure 15 Expression of natural targets of miR-122 and *let-7* in TuD-treated mice. C57BL/6 mice were administered with 1×10^{12} genome copies of control, anti-miR-122 TuD or anti-*let-7* TuD scAAV9 vector via tail vein injection. The animals were sacrificed four weeks later and total liver (left panel) or heart (right panel) RNA analyzed by qRT-PCR for representative endogenous targets of miR-122 (*Aldolase A, Cyclin G1, Tmed3, Hfe2*, and *Cat-1* mRNA) and *let-7* (*Kras, Hras, Nras,* and *Dicer* mRNA). The data are presented as the mean percentage (± standard deviation) of the expression in the mice treated with the control scAAV vector.

Figure 16 Change in cholesterol profiles of wild-type C57BL/6 and hypercholesterolemic mice ($LDLR^{-/-}$, $Apobec1^{-/-}$) after miR-122 antagonist treatment, relative to control mice. (**a**) Four-to-six week old male wild-type C57BL/6 mice were intravenously injected with 1×10^{12} genome copies of scAAV9 per mouse. Serum levels of total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in the treated C57B/6 were measured at different time points after injection. (**b**) The serum transaminases aspartate, aminotransferase (ASL) and alanine aminotransferase (ALT) were assayed to assess liver toxicity. (**c**) Adult male (n = 5 for scrambled and n=6 for anti-miR-122 TuD) and female (n = 9 for scrambled and n = 8 for anti-miR-122 TuD) $LDLR^{-/-}$, $Apobec1^{-/-}$ mice were administered 3×10^{11} genome copies of scAAV9 expressing antimiR-122 by tail vein injection. The changes in total cholesterol, HDL, and LDL, relative to the control, were measured one month later. The figure reports mean ± standard deviation.

Figure 17 Body weights of the study animals. The C57BL/6 wild-type mice treated with 1×10^{12} genome copies of scAAV9CBGluc (mock), scAAV9CBGlucTuDmiR-122 (anti-miR-122 TuD) or scAAV9CBGlucTuD*let-7* (anti-*let-7* TuD) via tail vein injection were weighed 10, 12, 14, 16 and 18 weeks later. The data are mean ± standard deviation.

DETAILED DESCRIPTION

Aspects of the invention are based on the discovery of miRNA inhibitors that are useful for treating and studying cholesterol-related disorders. In some aspects of the invention, a nucleic acid encoding a microRNA inhibitor is packaged in a recombinant AAV

(rAAV) for gene transfer to a subject. Recombinant AAVs comprising miRNA inhibitor genes of the invention are useful for therapeutic purposes as well as for research purposes. According to some aspects of the invention, methods are provided for treating a cholesterol-related disorder in a subject. In some embodiments, methods of the invention involve administering an effective amount of a rAAV to a subject. A rAAV may harbor at least one transgene that expresses a miRNA inhibitor that inhibits the expression of miR-122 in the subject. An exemplary miRNA inhibitor has a sequence as set forth in SEQ ID NO: 1.

Cholesterol-Related Disorders

As used herein, a "cholesterol-related disorder" is a condition or disease that results in a pathological change in cholesterol levels (*e.g.*, pathologically low or pathologically high) in a subject. A subject may be a mouse, a rat, a rabbit, a dog, a cat, a sheep, a pig, or a non-human primate, for example. A subject may be a human, *e.g.*, a subject having a cholesterol related disorder. In some embodiments, the subject is an animal model of a high cholesterol-related disorder. A cholesterol-related disorder may be associated with changes in levels of total serum cholesterol, serum HDL cholesterol, or serum LDL cholesterol. A cholesterol related disorder may also be associated with alterations in the ratio between serum LDL and HDL (*e.g.*, an LDL/HDL ratio). Examples of normal cholesterol ranges for different species are provided below in Table 1. As is evident from Table 1, normal ranges are species dependent. Cholesterol-related disorder associated with abnormally high levels of cholesterol are referred to herein as "high cholesterol-related disorders." For human subjects a high cholesterol-related disorder may be characterized by total serum cholesterol level greater than 200 mg/dl. For mouse subjects a high cholesterol-related disorder may be characterized by total serum cholesterol level greater than 100 mg/dl. For rat subjects a high cholesterol-related disorder may be characterized by total serum cholesterol level greater than or equal to 70 mg/dl. Other cholesterol levels that are abnormal will be apparent to the skilled artisan.

	Human	**Rat**	**Mouse**
Total cholesterol (mg/dL)	**140~199**	**50~70**	**~100**
LDL (mg/dL)	**105~120**	**7~11**	**5~20**
HDL (mg/dL)	**30~59**	**29~40**	**50~100**

Table 1. Exemplary ranges of normal cholesterol levels.

Examples of cholesterol-related disorders that may be treated according to aspects of the invention include, but are not limited to, Type I, Type II(a and b), Type III, Type IV, and Type V Hyperlipoproteinemia. Further disorders that may be treated according to aspects of the invention include cholesterol-related disorders associated with diabetes mellitus, metabolic syndrome, kidney disease (nephrotic syndrome), hypothyroidism, Cushing's syndrome, anorexia nervosa, sleep deprivation, Zieve's syndrome, antiretroviral drugs, diet, high body weight, or low physical activity. Other cholesterol-related disorders will be apparent to the skilled artisan.

Certain cholesterol-related disorders that may be treated according to aspects of the invention are disorders of a genetic origin (*e.g.*, inherited, arising from somatic mutations). Familial hypercholesterolemia (FH) (Type II Hyperlipoproteinemia), for example, is a cholesterol-related disorders of genetic origin characterized by high cholesterol levels, specifically very high low-density lipoprotein (LDL) levels, in the blood and early cardiovascular disease. Many subjects with FH have mutations in the LDLR gene that encodes the LDL receptor protein, which normally removes LDL from the circulation, or apolipoprotein B (ApoB), which is the part of LDL that binds with the receptor; mutations in other genes are rare. Subjects who have one abnormal copy (are heterozygous) of the LDLR gene may have premature cardiovascular disease at the age of 30 to 40.

MiRNA Inhibitors

Micro RNAs (miRNAs) appear to play a role in regulating a broad range of cellular processes, and changes in miRNA expression have been implicated in human disease. It is understood that microRNAs have a role in the development and progression of certain cholesterol-related disorders. The most abundant miRNA in the liver, miR-122 regulates cholesterol metabolism by an unknown mechanism and does not directly target LDLR mRNA. Although miR-122 represents a potential therapeutic target for high cholesterol-related disorders, the prospect of therapeutically effective inhibitors of miR-122 has been largely unfulfilled.

As used herein, the term "miRNA Inhibitor" refers to an agent that blocks miRNA expression, processing and/or function. A variety of miRNA Inhibitor have been disclosed in the art. Non-limiting examples of miRNA inhibitors include but are not limited to microRNA specific antisense, microRNA sponges, tough decoy RNAs (TuD RNAs) and microRNA oligonucleotides (double-stranded, hairpin, short oligonucleotides) that inhibit miRNA

interaction with a Drosha complex. (See, *e.g.*, Ebert, M.S. Nature Methods, Epub August, 12, 2007; Takeshi Haraguchi, et al., Nucleic Acids Research, 2009, Vol. 37, No. 6 e43, the contents of which relating to TuD RNAs are incorporated herein by reference).

Molecular sensing system for miRNA Inhibitors

A molecular sensing system was designed to quantitatively evaluate the inhibitory function of different miRNA inhibitor designs and to enable discovery of miRNA inhibitors having superior properties compared with miRNA inhibitors of the art (See Figure 1 for a schematic of a molecular sensing system). Various miRNA inhibitors were developed and tested using this system (See, *e.g.*, Example 1). According to some aspects of the invention a miRNA inhibitor of miR-122 is identified that effectively reduces serum cholesterol levels.

A molecular sensing system of the invention typically includes components for expressing RNA transcripts of a reporter gene (*e.g.*, a protein coding gene, *e.g.*, EGFP, Luciferase), the expression of which is sensitive to a miRNA that binds to the RNA transcript. Typically, the RNA transcript is an mRNA transcript encoding a protein. Thus, reporter gene activity is often assessed by detecting levels of a protein encoded by an mRNA transcript of the transgene. However, the RNA transcript of the reporter gene may itself serve as a reporter of transgene activity. For example, the RNA may be detected using any one of a variety of standard RNA detection strategies, *e.g.* RT-PCR, and thus, may serve as a reporter for activity of the transgene. Typically, the RNA transcript of the transgene bears one or more miRNA binding sites. Thus, when expressed in a cell, RNA transcripts of a molecular sensing system are typically sensitive to the presence of miRNA molecules of the cell that bind to them at miRNA binding sites. The miRNA binding sites are typically in the 3' end of the transcript. However, a miRNA binding site may be in a coding region or in any untranslated region of the transgene provided that when a miRNA binds to the site in a cell having a functional miRNA gene silencing pathway, or a *in vitro* system that recapitulates miRNA activity, expression of the transcript is inhibited. The molecule sensing system also typically comprises components for expressing a test miRNA inhibitor. When mRNA transcripts bearing binding sites for a miRNA are expressed in the presence of the miRNA, the miRNA hybridizes to the binding sites and inhibits expression of a reporter protein encoded by the mRNA. However, when a miRNA inhibitor is expressed that blocks function of the miRNA, expression of the reporter protein is not inhibited (or inhibition of expression is attenuated). Thus, molecular sensing systems of the invention enable efficient screening and identification

of miRNA inhibitors with effective inhibitory properties based on levels of reporter gene expression.

A molecule sensing system often includes a nucleic acid vector comprising a promoter operably linked with a transgene that is regulated by a miRNA and a promoter operably linked with an miRNA inhibitor coding region. The transgene of the nucleic acid vector typically includes, at a minimum, a protein coding region (*e.g.*, a reporter protein coding region) and at least one binding site of a miRNA. The protein coding region may encode a reporter protein such as, for example, a fluorescent protein, (*e.g.*, GFP, dsRed, etc.) luciferase, β-galactosidase, secreted alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase (CAT), and β-lactamase. The promoter for the transgene and the promoter for the miRNA inhibitor coding region may be the same promoter or may be different promoters. The promoter for the transgene is typically a RNA Polymerase II promoter. The promoter for the miRNA inhibitor may be a RNA Polymerase II promoter or an RNA Polymerase III promoter (*e.g.*, a U6 promoter).

The skilled artisan will appreciate that the promoter operably linked with the transgene may be positioned anywhere within the nucleic acid vector provided that the transgene is capable of being expressed in an appropriate expression system, *e.g.*, in a cell or an *in vitro* transcription/translation system. Similarly, the skilled artisan will appreciate that the promoter operably linked with the miRNA inhibitor coding region may be positioned anywhere within the nucleic acid vector provided that the miRNA inhibitor coding region is capable of being expressed in an appropriate expression system, *e.g.*, in a cell or an *in vitro* transcription/translation system. For example, the second promoter operably linked with a miRNA inhibitor coding region may be positioned upstream of the first promoter operably linked with the transgene (5-prime relative to the first promoter operably linked with the transgene.) The second promoter operably linked with a miRNA inhibitor coding region may be positioned downstream of the first promoter operably linked with the transgene (3-prime relative to the first promoter operably linked with the transgene.) The second promoter operably linked with a miRNA inhibitor coding region may be positioned between the first promoter and the transgene coding region (*e.g.*, within an intron). The second promoter operably linked with a miRNA inhibitor coding region may be positioned within any intron of the transgene. The second promoter operably linked with a miRNA inhibitor coding region may be positioned within a untranslated region upstream of the transgene coding region (*e.g.*, a 5'-UTR) or downstream of the transgene coding region (*e.g.*, a 3'-UTR).

A molecule sensing system may include, for example, a nucleic acid vector comprising a first promoter operably linked with a transgene that is regulated by a test miRNA and a second promoter operably linked with a miRNA inhibitor coding region. The nucleic acid vector may be a recombinant viral genome. For example, the nucleic acid vector may be a recombinant AAV vector. Accordingly, the nucleic acid vector further may include a pair of inverted terminal repeats that flank the promoter operably linked with transgene. The pair of inverted terminal repeats may further flank the promoter operably linked with the miRNA inhibitor coding region.

Methods are provided for assessing the effectiveness of a miRNA inhibitor using a molecular sensing system of the invention. The methods typically involve (a) transfecting a cell with a nucleic acid vector, which comprises a first promoter operably linked with a transgene that comprises a protein coding region and at least one binding site of a miRNA and a second promoter operably linked with a coding region for a miRNA inhibitor that hybridizes with the miRNA, and (b) determining the level of expression of the protein encoded by the protein coding region in the cell. The level of expression of the protein is indicative of the effectiveness of the miRNA inhibitor. For example, when the nucleic acid vector is transfected in a cell that expresses the miRNA, the miRNA will bind to its cognate binding site(s) in the mRNA transcribed from the transgene and inhibit expression of the mRNA. If the miRNA inhibitor is effective, it will block (or decrease) the activity of the miRNA, *e.g.*, by hybridizing with the miRNA, and relieve (or attenuate) repression of expression of the mRNA. Changes in expression of the mRNA are typically observed by assessing levels of the reporter protein encoded by the mRNA. Thus, different miRNA inhibitors can be compared based on reporter protein levels. As will be appreciated by the skilled artisan, the system can be tuned in various ways to identify inhibitors having desired levels of effectiveness. For example, the quality of the miRNA binding site on the transgene mRNA can be designed or selected. High quality binding sites, *e.g.*, binding sites that bind to the test miRNA with high affinity can be designed or selected. Binding sites can be designed *de novo* or selected from miRNA bindings sites of known genes (*e.g.*, an miR-122 binding site on Cyclin G may be selected). The number of miRNA binding sites in the transgene mRNA can also be altered. For example, multiple binding sites can be used or a single binding site can be used. By adjusting parameters such as the affinity of the miRNA for binding to its miRNA and the number of bindings sites, it becomes possible to increase or decrease the stringency with which miRNA inhibitors are selected. For example, high quality miRNA inhibitors can be

selected by using a transgene having multiple high-quality bindings sites for a test miRNA. The molecule sensing system may be used in an *in vitro* expression system or in cells.

The level of the miRNA is another example of a parameter that can be modulated to increase or decrease the stringency with which miRNA inhibitors are selected. Thus, the methods may further comprise contacting cells with the miRNA or adding miRNA to an *in vitro* expression system. Multiple experiments may be performed, *e.g.*, in parallel, using different doses of the miRNA to enable an evaluation of the dose dependent inhibition properties of the miRNA inhibitors.

Any of a variety of control values or experiments may be obtained or performed to assess the effectiveness of a test miRNA inhibitor. The methods may comprise (a) transfecting a first cell with a nucleic acid vector of a molecular sensing system, wherein the miRNA inhibitor coding region of the vector encodes the miRNA inhibitor; (b) transfecting a second cell with the nucleic acid vector, wherein levels of the test miRNA are lower in the second cell compared with the first cell; and (c) comparing the level of expression of the protein encoded by the protein coding region in the first cell with the level of expression of the protein encoded by the protein coding region in the second cell, wherein the results of the comparison in (c) are indicative of the effectiveness of the miRNA inhibitor. The methods may comprise (a) transfecting a cell with any one of the foregoing nucleic acid vectors, wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; (b) determining a first level of expression of the protein encoded by the protein coding region in the cell; (c) contacting the cell with the test miRNA; (d) determining a second level of expression of the protein encoded by the protein coding region in the cell; and (e) comparing the first level of expression of the protein with the second level of expression, wherein the results of the comparison in (e) are indicative of the effectiveness of the miRNA inhibitor.

MiRNA Inhibitor Structure

Aspects of the invention are based on the discovery of miRNA inhibitors that target miR-122 and block its function. For example, high quality miRNA inhibitors have been discovered using a molecular sensing system of the invention.

The typical miRNA inhibitor of the invention is a nucleic acid molecule that comprises at least one miRNA binding site, *e.g.*, an miR-122 binding site. The miRNA inhibitors may comprise 1 miRNA binding site, 2 miRNA binding sites, 3 miRNA binding sites, 4 miRNA binding sites, 5 miRNA binding sites, 6 miRNA binding sites, 7 miRNA binding sites, 8

miRNA binding sites, 9 miRNA binding sites, 10 miRNA binding sites, or more miRNA binding sites. As used herein, the term "miRNA binding site," with reference to a miRNA inhibitor, refers to a sequence of nucleotides in a miRNA inhibitor that are sufficiently complementary with a sequence of nucleotides in a miRNA to effect base pairing between the miRNA inhibitor and the miRNA. Typically, a miRNA binding site comprises a sequence of nucleotides that are sufficiently complementary with a sequence of nucleotides in a miRNA to effect base pairing between the miRNA inhibitor and to thereby inhibit binding of the miRNA to a target mRNA.

As used herein the term "complementary" or "complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another RNA sequence by either traditional Watson-Crick or other non-traditional base pairing. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, *e.g.*, miRNA inhibition. Determination of binding free energies for nucleic acid molecules is well known in the art (see, *e.g.*, Turner et al., 1987, CSH Symp. Quant. Biol. LII pp. 123 133; Frier et al., 1986, Proc. Nat. Acad. Sci. USA 83:9373 9377; Turner et al., 1987, J. Am. Chem. Soc. 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule which can form hydrogen bonds (*e.g.*, Watson-Crick base pairing) with a second nucleic acid sequence (*e.g.*, 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. In some embodiments the nucleic acids have 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% complementarity.

For a miRNA inhibitor having two miRNA binding sites, the first miRNA binding site and the second miRNA binding site may be complementary, *e.g.*, at a sequence of 2 to 10 nucleotides in length. In one example, the first miRNA binding and the second miRNA binding site are complementary at a sequence of 4 nucleotides in length. Each miRNA binding site of a miRNA inhibitor may be any of a variety of lengths. For example, the miRNA binding site of a miRNA inhibitor may be 5 nucleotides to 35 nucleotides, 10 nucleotides to 30 nucleotides, or 15 nucleotides to 25 nucleotides. Typically the length of the miRNA binding site depends on the length and/or structure of the miRNA to which it binds.

Often a miRNA binding site of a miRNA inhibitor of the invention is flanked by one or more stem sequence. As used herein the term "stem sequence" refers to a sequence of a nucleic acid that results in intramolecular base pairing. In some embodiments, stem sequences are not complementary with a target miRNA. Intramolecular base pairing may occur when two stem sequence regions of a miRNA inhibitor, usually palindromic sequences, base-pair to form a double helix, which may end in an unpaired loop. Thus, based pairing may form within a stem sequence or between two stem sequences. A stem sequence may be of a variety of lengths. For example, a stem sequence may be in range 3 nucleotides to 200 nucleotides, 3 nucleotides to 100 nucleotides, 3 nucleotides to 50, 3 nucleotides to 25 nucleotides, 10 nucleotides to 20 nucleotides, 20 nucleotides to 30 nucleotides, 30 nucleotides to 40 nucleotides, 40 nucleotides to 50 nucleotides, or 50 nucleotides to 100 nucleotides. A stem sequence may be up to 5 nucleotides, up to 10 nucleotides, up to 20 nucleotides, up to 50 nucleotides, up to 100 nucleotides, up to 200 nucleotides, or more. Linker sequences may also be included in a miRNA inhibitor. The miRNA inhibitor may comprise a first miRNA binding site and a second miRNA binding site, each binding site flanked by two stem sequences. A first stem sequence may flank the first miRNA binding site at its 5'-end, a second stem sequence may flank the first miRNA binding site at its 3'-end and the second miRNA binding site at its 5'-end, and a third stem sequence may flank the second miRNA binding site at its 3'-end. The skilled artisan will readily envision other configurations of binding sites and flanking stem sequences.

The miRNA binding site of a miRNA inhibitor of the invention may comprise a non-binding, central portion that is not complementary with the target miRNA (*e.g.*, miR-122), flanked by two portions that are complementary with the target miRNA. A non-binding, central portion that is not complementary with the target miRNA need not be perfectly centered within the miRNA binding site. For example, a non-binding central portion may be flanked on either side by portions that are complementary with the target miRNA that are of different lengths. A miRNA inhibitor of the invention may comprise multiple miRNA binding sites that have a non-binding, central portion that is not complementary with the target miRNA. The non-binding, central portion of a miRNA binding site may have any of a variety of lengths. For example, a non-binding, central portion of a miRNA binding site may be in a range of 1 nucleotide to 20 nucleotides, 1 nucleotide to 10 nucleotides, 1 nucleotide to 5 nucleotides. The non-binding, central portion of a miRNA binding site may have a length in a range of 3 to 5 nucleotides. In one example, the non-binding, central portion of a miRNA

binding site has a length of 4 nucleotides. The length of the non-binding, central portion will typically depend on the length of the miRNA binding site.

Often the non-binding, central portion of a first miRNA binding site is at least partially complementary with the non-binding, central portion of a second miRNA binding site of the inhibitor. Thus, two binding sites of an inhibitor may base pair (hybridize) with each other. The non-binding, central portion of a first miRNA binding site of an inhibitor may be complementary with the non-binding, central portion of a second miRNA binding site of an inhibitor at, for example, 2 nucleotides to 10 nucleotides, depending on the length of the binding site and the non-binding central portion. The non-binding, central portion of a first miRNA binding site of an inhibitor may be complementary with the non-binding, central portion of a second miRNA binding site at, for example, 2 nucleotides, 3 nucleotides, 4 nucleotides, 5 nucleotides, 10 nucleotides, or more nucleotides, typically depending on the length of the binding site and the non-binding central portion.

Some aspects of this invention provide miRNA inhibitors that target a plurality of miRNAs. In some embodiments, targeting a plurality of miRNAs circumvents the problem of inhibition of an individual miRNA being compensated for by related miRNAs. In some embodiments, the plurality of miRNAs belong to a family of miRNAs, for example, the let-7 family. In some embodiments, the plurality of miRNAs share at least some sequence identity. For example, in some embodiments, the plurality of miRNAs each comprise at least one stretch of 5 or more nucleotides that is identical across all of the plurality of miRNAs. In some embodiments, the plurality of miRNAs each comprise at least one stretch of 5 or more nucleotides that is at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identical to the consensus sequence of that stretch of nucleotides of the plurality of target miRNAs.

The term "consensus sequence," as used herein, refers to a sequence of nucleotides that reflects the most common nucleotide shared by multiple nucleotide sequences at a specific position. In some embodiments, the multiple nucleotide sequences are related nucleotide sequences, for example, sequences of members of the same miRNA family. In some embodiments, a consensus sequence is obtained by aligning two or more sequences and determining the nucleotide most commonly found or most abundant in the aligned sequences at a particular position. Methods and algorithms for sequence alignment for obtaining consensus sequences from a plurality of sequences are well known to those of skill in the art and the invention is not limited in this respect.

In some embodiments, the miRNA inhibitor targeting a plurality of miRNAs is TuD comprising at least one miRNA binding site complementary to a consensus sequence of the plurality of miRNAs. In some embodiments, the consensus sequence is at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 nucleotides in length. In some embodiments, the miRNA inhibitor comprises a first miRNA binding site and a second miRNA binding site, wherein a first stem sequence flanks the first miRNA binding site at its 5'-end, a second stem sequence flanks the first miRNA binding site at its 3'-end and the second miRNA binding site at its 5'-end, and a third stem sequence flanks the second miRNA binding site at its 3'-end, wherein at least one of the miRNA binding sites comprises a nucleotide sequence complementary to a consensus sequence of the plurality of target miRNAs. In some embodiments, the first and the second miRNA binding sites are complementary to a consensus sequence of the plurality of target miRNAs. In some embodiments, the first and/or the second miRNA binding site is at least 7-%, at least 80%, at least 90%, at least 95%, or at least 98% complementary to a consensus sequence of the plurality of target miRNAs. In some embodiments, the consensus sequence the first miRNA binding site is complementary to is directly adjacent to the consensus sequence the second miRNA binding site is complementary to.

In some embodiments, a miRNA inhibitor is provided that targets a plurality of let-7 family member miRNAs. In some embodiments, the miRNA inhibitor comprises a sequence of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, or 26, contiguous nucleotides of SEQ ID NO: 18. In some embodiments, the miRNA inhibitor comprises or consists of the nucleotide sequence of SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. In some embodiments, methods are provided that comprise contacting a cell with an miRNA inhibitor. The cell may be *in vitro* or may be *in vivo*. Accordingly, in some embodiments, the methods involve adding a miRNA inhibitor to a culture of cells *in vitro*. In other embodiments, the methods involve administering a miRNA inhibitor to a subject.

Some aspects of this invention provide a method of generating a miRNA inhibitor targeting a plurality of miRNAs, wherein the method comprises obtaining a consensus sequence of the plurality of target miRNAs, and generating a miRNA inhibitor, for example, a miRNA inhibitor described herein (e.g., a TuD), comprising a miRNA binding site able to

bind to the consensus sequence, and, thus, targeting the plurality of miRNAs. In some embodiments, the miRNA inhibitor so generated comprises a first miRNA binding site and a second miRNA binding site, wherein a first stem sequence flanks the first miRNA binding site at its 5'-end, a second stem sequence flanks the first miRNA binding site at its 3'-end and the second miRNA binding site at its 5'-end, and a third stem sequence flanks the second miRNA binding site at its 3'-end, wherein the miRNA inhibitor comprises a nucleotide sequence complementary to the consensus sequence of the plurality of target miRNAs. In some embodiments, the method further comprises synthesizing the miRNA inhibitor targeting a plurality of miRNAs.

Recombinant AAVs

It has been discovered that the miRNA inhibitors of the invention when expressed from a recombinant AAV vector achieve long-term miRNA inhibitory effects in a subject. For example, it has been discovered that a miRNA inhibitor against miR-122 delivered using a rAAV to a normal subject (who does not have a cholesterol-related disorder) significantly reduces total serum cholesterol in the subject for a sustained period of time, *e.g.*, up to at least 14 weeks. It has further been discovered that a miRNA inhibitor against miR-122 delivered using a rAAV to a subject having a high cholesterol-related disorder also significantly reduces total serum cholesterol in the subject for a sustained period of time, *e.g.*, up to at least 14 weeks.

AAVs are natural inhabitants in mammals. AAVs isolated from mammals, particularly non-human primates, are useful for creating gene transfer vectors for clinical development and human gene therapy applications. In aspects of the invention, a recombinant AAV9 achieves efficient and stable miR-122 antagonism in normal C57BL/6 mice by expressing an optimized miR-122 inhibitor (also referred to herein as an miR-122 antagonist (Antag)). A single intravenous injection of a rAAV9 comprising a rAAV vector encoding an miR-122 inhibitor (rAAV9-miR-122Antag) produced an significant decrease in the level of mature miR-122 and significant up-regulation of miR-122 target genes. A reduction in total serum cholesterol, HDL, and LDL of up to about 50% was observed in a normal subject who was fed a regular diet.

In some aspects, the invention provides isolated AAVs. As used herein with respect to AAVs, the term "isolated" refers to an AAV that has been isolated from its natural environment (*e.g.*, from a host cell, tissue, or subject) or artificially produced. Isolated AAVs

may be produced using recombinant methods. Such AAVs are referred to herein as "recombinant AAVs". Recombinant AAVs (rAAVs) preferably have tissue-specific targeting capabilities, such that a transgene of the rAAV will be delivered specifically to one or more predetermined tissue(s). The AAV capsid is an important element in determining these tissue-specific targeting capabilities. Thus, a rAAV having a capsid appropriate for the tissue being targeted can be selected. Typically, the rAAV has a capsid that has a tropism for (that targets) liver tissue, particularly hepatocytes of liver tissue. For example, the rAAV capsid may be of the AAV9 serotype, which has a sequence as set forth in SEQ ID NO: 3, or a variant thereof. The rAAV has a capsid of the AAV9 serotype variant, Csp-3, which has a sequence as set forth in SEQ ID NO: 4. Examples of AAV9 serotype variants are disclosed in U.S. Provisional Application Serial Number 61/182,084, filed May 28, 2009, the contents of which relating to AAV capsid sequences are incorporated herein by reference. Still, in some embodiments the AAV serotype is selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, and AAVrh.10. In other embodiments the AAV serotype is a variant of an AAV serotype is selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, and AAVrh.10.

>gi|46487805|gb|AAS99264.1| capsid protein VP1 [Adeno-associated virus 9]
(SEQ ID NO: 3)
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGP
GNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSF
GGNLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPA
KKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSS
SGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIAN
NLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSF
YCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTIN
GSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGASS
WALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITNEE
EIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGPIW
AKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQ
VSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL

>capsid protein VP1 [Adeno-associated virus] CSp3
(SEQ ID NO: 4)
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGP
GNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSF
GGNLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPA
KKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTIASGGGAPVADNNEGADGVGSSS
GNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKRISNSTSGGSSNDNAYFGYSTPW
GYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIRVKEVTDNNGVKTITNNL

TSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRSSFYC
LEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKTINGS
GQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTRNNNSEFAWPGASSWA
LNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITNEEEIK
TTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGPIWAKI
PHTDGNFHPSPLMGGFGVKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSV
EIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL

Recombinant AAVs: Production Methods

Methods for obtaining recombinant AAVs having a desired capsid protein are well known in the art. (See, for example, US 2003/0138772, the contents of which are incorporated herein by reference in their entirety). Typically the methods involve culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein or fragment thereof; a functional *rep* gene; a recombinant AAV vector composed of, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the recombinant AAV vector into the AAV capsid proteins.

The components to be cultured in the host cell to package a rAAV vector in an AAV capsid may be provided to the host cell in *trans*. Alternatively, any one or more of the required components (*e.g.*, recombinant AAV vector, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are provided herein, in the discussion of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contain the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

The recombinant AAV vector, rep sequences, cap sequences, and helper functions required for producing the rAAV of the invention may be delivered to the packaging host cell using any appropriate genetic element (vector). The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any [illegible] n are known to those with skill in nucleic acid manipulation and

include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.*, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present invention. See, *e.g.*, K. Fisher et al, J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745.

In some embodiments, recombinant AAVs may be produced using the triple transfection method (described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with an recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (*i.e.*, rep and cap), which function in *trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (*i.e.*, AAV virions containing functional rep and cap genes). Non-limiting examples of vectors suitable for use with the present invention include pHLP19, described in U.S. Pat. No. 6,001,650 and pRep6cap6 vector, described in U.S. Pat. No. 6,156,303, the entirety of both incorporated by reference herein. The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpes virus (other than herpes simplex virus type-1), and vaccinia virus.

In some aspects, the invention provides transfected host cells. The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous nucleic acids, such as a nucleotide integration vector and other nucleic acid

molecules, into suitable host cells. Transfection may be achieve for example by infecting a cell with a rAAV harboring a rAAV vector.

A "host cell" refers to any cell that harbors, or is capable of harboring, a substance of interest. Often a host cell is a mammalian cell. A host cell may be used as a recipient of an AAV helper construct, an AAV transgene plasmid, *e.g.*, comprising a promoter operably linked with a miRNA inhibitor, an accessory function vector, or other transfer DNA associated with the production of recombinant AAVs. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein may refer to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

As used herein, the term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, *etc.*, which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression

of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some embodiments, expression includes transcription of the nucleic acid, for example, to generate a biologically-active polypeptide product or inhibitory RNA (*e.g.*, shRNA, miRNA, miRNA inhibitor) from a transcribed gene.

The foregoing methods for packaging recombinant vectors in desired AAV capsids to produce the rAAVs of the invention are not meant to be limiting and other suitable methods will be apparent to the skilled artisan.

Recombinant AAV vectors

"Recombinant AAV (rAAV) vectors" of the invention are typically composed of, at a minimum, a transgene, *e.g.*, encoding a miRNA inhibitor or a nucleic acid of a molecular sensing system, and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). It is this recombinant AAV vector which is packaged into a capsid protein and delivered to a selected target cell. In some embodiments, the transgene is a nucleic acid sequence, heterologous to the vector sequences, which encodes a miRNA inhibitor. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a cell of a target tissue. Recombinant AAV based vectors may be developed for targeting the miRNA inhibitors to liver tissue to interfere with miR-122 function and reduced cholesterol levels. Recombinant AAV based vectors may also be developed for targeting a nucleic vector of a molecular sensing system to cell for evaluating or screening miRNA inhibitors in the cell.

The AAV sequences of the vector typically comprise the cis-acting 5' and 3' inverted terminal repeat sequences (See, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, *e.g.*, texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher et al., J Virol., 70:520 532 (1996)). An example of such a molecule employed in the present invention is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by

the 5' and 3' AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including presently identified mammalian AAV types.

In addition to the major elements identified above for the recombinant AAV vector, the vector also includes conventional control elements necessary which are operably linked to the transgene in a manner which permits its transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced by the invention. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

As used herein, a nucleic acid sequence (*e.g.*, coding sequence) and regulatory sequences are said to be "operably" linked when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. If it is desired that the nucleic acid sequences be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not interfere with the ability of the promoter region to direct the transcription of the coding sequences or interfere with the function of the corresponding RNA transcript. Thus, a promoter region would be operably linked to a nucleic acid sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might become a functional RNA molecule (*e.g.*, a properly folded miRNA inhibitor).

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*.) are well known in the art. Often, a miRNA inhibitor is expressed from a polymerase III promoter, such as, for example, a U6

promoter. However, other appropriate promoters, *e.g.*, RNA polymerase II promoters, may be used.

Recombinant AAV Administration Methods

The rAAVs may be delivered to a subject in compositions according to any appropriate methods known in the art. The rAAV, preferably suspended in a physiologically compatible carrier (*i.e.*, in a composition), may be administered to a subject, such as, for example, a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-human primate (e.g., Macaque).

The rAAVs are administered in sufficient amounts to transfect the cells of a desired tissue and to provide sufficient levels of gene transfer and expression without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the selected organ (*e.g.*, intraportal delivery to the liver), oral, inhalation (including intranasal and intratracheal delivery), intraocular, intravenous, intramuscular, subcutaneous, intradermal, intratumoral, and other parental routes of administration. In certain circumstances it will be desirable to deliver the rAAV-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraopancreatically, intranasally, parenterally, intravenously, intramuscularly, intrathecally, or orally, intraperitoneally, or by inhalation. In some embodiments, the administration modalities as described in U.S. Pat. Nos. 5,543,158; 5,641,515 and 5,399,363 (each specifically incorporated herein by reference in its entirety) may be used to deliver rAAVs.

Delivery of the rAAVs to a mammalian subject may be by intravenous injection. In some embodiments, the mode of administration of rAAVs is by portal vein injection. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit. In some embodiments, administration of rAAVs into the bloodstream is by way of isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV virions. A variant of the isolated limb perfusion technique, described in U.S. Pat. No. 6,177,403, can also be employed by the skilled artisan to administer the rAAVs into the vasculature of an isolated limb to potentially enhance transduction into muscle cells or tissue. Routes of administration may be combined, if desired.

Moreover, in certain instances, it may be desirable to deliver the virions to the CNS of a subject. By "CNS" is meant all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cereobrospinal fluid (CSF), interstitial spaces, bone, cartilage and the like. Recombinant AAVs may be delivered directly to the CNS or brain by injection into, *e.g.*, the ventricular region, as well as to the striatum (*e.g.*, the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, *e.g.*, Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000).

The compositions of the invention may comprise a rAAV alone, or in combination with one or more other viruses (*e.g.*, a second rAAV encoding having one or more different transgenes, *e.g.*, one or more different miRNA inhibitors). In some embodiments, a compositions comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different rAAVs each having one or more different transgenes.

Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the rAAV is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

Optionally, the compositions of the invention may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The dose of rAAV virions required to achieve a particular "therapeutic effect," *e.g.*, the units of dose in genome copies/per kilogram of body weight (GC/kg), will vary based on several factors including, but not limited to: the route of rAAV virion administration, the level of gene or RNA expression required to achieve a therapeutic effect, the specific disease or disorder being treated, and the stability of the gene or RNA product. One of skill in the art can readily determine a rAAV virion dose range to treat a subject having a particular disease or

disorder based on the aforementioned factors, as well as other factors that are well known in the art.

An effective amount of a rAAV is an amount sufficient to target infect an animal, target a desired tissue. In some embodiments, an effective amount of a rAAV is an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend primarily on factors such as the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among animal and tissue. For example, a effective amount of the rAAV is generally in the range of from about 1 ml to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies. In some cases, a dosage between about 10^{11} to 10^{12} rAAV genome copies is appropriate. In certain preferred embodiments, 10^{12} rAAV genome copies is effective to target heart, liver, and pancreas tissues. In certain embodiments, the dosage of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, or 10^{14} genome copies per kg. In certain embodiments, the dosage of rAAV is 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, or 10^{15} genome copies per subject. In some cases, stable transgenic animals are produced by multiple doses of a rAAV.

In some embodiments, rAAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high rAAV concentrations are present (*e.g.*, ~10^{13} GC/ml or more). Methods for reducing aggregation of rAAVs are well known in the art and, include, for example, addition of surfactants, pH adjustment, salt concentration adjustment, *etc.* (See, *e.g.*, Wright FR, et al., Molecular Therapy (2005) 12, 171–178, the contents of which are incorporated herein by reference.)

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing

such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

Sterile injectable solutions are prepared by incorporating the active rAAV in the required amount in the appropriate solvent with various of the other ingredients enumerated

herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The rAAV compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered transgenes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art. Recently,

liposomes were developed with improved serum stability and circulation half-times (U.S. Pat. No. 5,741,516). Further, various methods of liposome and liposome like preparations as potential drug carriers have been described (; U.S. Pat. Nos. 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures. In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs, radiotherapeutic agents, viruses, transcription factors and allosteric effectors into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 .ANG., containing an aqueous solution in the core.

Alternatively, nanocapsule formulations of the rAAV may be used. Nanocapsules can generally entrap substances in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use.

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the rAAV compositions to a host. Sonophoresis (e.g., ultrasound) has been used and described in U.S. Pat. No. 5,656,016 as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U.S. Pat. No. 5,779,708), microchip devices (U.S. Pat. No. 5,797,898), ophthalmic formulations (Bourlais et al., 1998), transdermal matrices (U.S. Pat. Nos. 5,770,219 and 5,783,208) and feedback-controlled delivery (U.S. Pat. No. 5,697,899).

Kits and Related Compositions

The agents described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or

research applications. A kit may include one or more containers housing the components of the invention and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended application and the proper use of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the invention. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, *etc.*), Internet, and/or web-based communications, *etc.* The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for animal administration.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. In some embodiments, the kit comprises a container(s) housing agents (components) of a molecular sensing system. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, ampule or other container. The kit may have one or more or all of the components required to administer the agents to an animal, such as a syringe, topical

application devices, or iv needle tubing and bag, particularly in the case of the kits for producing specific somatic animal models.

EXAMPLES

Example 1: rAAV-mediated delivery of microRNA scavengers leads to efficient and stable knock-down of cognate microRNA, upregulation of their natural target genes and phenotypic changes in mice

The use of rAAV for the delivery of miRNA antagonists (miR-Antags) in adult mice was investigated. Different designs of vector backbone (ss versus sc), promoter (Pol II versus Pol III) and miRNA antagonist (Sponge, Zip, TuD, etc.) were evaluated for efficient somatic inhibition of specific miRNAs. Different designs of miRNA antagonists (inhibitors) were also evaluated, *e.g.*, bulged binding sites, multiple-tandem copy sponges, *etc.* MiR-122, which has been reported to regulate cholesterol biosynthesis in the liver, and an anti-oncogenic miRNA, Let-7, were used as targets for inhibition. In order to select high function inhibitors, a chemiluminescent miRNA sensor was developed (See Figure 1). The chemiluminescent miRNA sensor contained a Polymerase II promoter driving expression of a reporter gene in a rAAV vector. The reporter gene had an intron just downstream of the promoter and a series of miRNA binding sites (sponges) upstream of a poly-A tail. The polymerase II promoter and reporter gene were flanked by inverted terminal repeat sequences. A U6 promoter driving expression of the test miRNA inhibitor was present in the intron of the reporter gene. Thus, the miRNA sensor comprises dual miRNA regulators for sequential repression and de-repression of the reporter gene and target validation.

The effectiveness of miR-122 tough decoy RNA designs were assessed. 293 cells were infected with a miRNA sensor encoding β-galactosidase and expressing miR122 tough decoy RNAs. A control miRNA sensor was also transfected that did not express miR122 decoys RNAs. The test and control miRNA sensors each had 3 miR-122 binding sites. The 293 cells were transfected with 0 ng, 50 ng, 100 ng, 200 ng, and 400 ng. LacZ staining was performed using standard techniques to evaluate reporter gene expression. A dose dependent inhibition of reporter gene expression was observed in the control miRNA sensor. However, the test miRNA sensor which expressed miR122 inhibitor exhibited significant attenuation of inhibition of the reporter gene expression at all doses. In contrast, cells infected with a miRNA sensor encoding β-galactosidase and expressing miR122 sponge RNAs did not attenuate reporter gene expression compared with control miRNA sensors. Thus, the TuD

miR122 design was superior to the sponge design. Similar experiments where performed in Huh7 cells which expressed steady-state levels of about 1.6 x 10^4 miR-122 molecules per cell. In Huh7 cells, the effect of miRNA binding site number was evaluated. The test and control miRNA sensors each had either 1 or 3 miR-122 binding sites. It was found that TuD miR-122 RNA (SEQ ID NO: 1) completely rescued the down-regulation associated with one copy of a miR-122 binding site behind the LacZ reporter gene in Huh-7 cells. Different combinations of promoters (Pol II and Pol III) and miRNA inhibitors were evaluated. Polymerase III driving expression of TuD miR-122 inhibitors has superior results in both 293 and Huh-7 cells (Figure 2A and B). A similar miRNA sensor having a firefly luciferase (Fluc) reporter gene was developed and tested in Huh-7 cells. Again, TuD miR-122 RNA efficiently rescued the down-regulation of Fluc mediate by miR-122 binding sites in Huh-7 cells.

Mice (adult B6) infected with rAAV (Serotype 9) harboring TuD miR-122 inhibitor genes (for up to 7 weeks post infection) exhibited no adverse effects on liver function, as assessed by liver enzymes levels. Effective induction of miR-122 target genes was observed in mice infected with rAAV (Serotype 9) harboring TuD miR-122 inhibitor genes after 1 month post infection, compared with control mice which were infected with rAAV9 harboring scrambled inhibitors. The target genes evaluated include Aldolase A, Cyclin G1, Tmed3 and Hfe2. MiR-122 inhibitors delivered by rAAV9 had no effect on these target genes in the mouse heart, the cells of which do not express miR122. A single IV injection of rAAVmiR-122-Antag to C57BL/6 mice produced an 80% decrease in the level of mature miR-122 (Figure 2C) and a 3-fold increase in the mRNA levels of miR-122 target genes. Inhibition of miR-122 reduced total serum cholesterol, HDL, and LDL by 50% in mice fed a regular diet. The sequence and secondary structure of the TuD miR-122 inhibitor is shown in Figures 3A and 3B, respectively.

Similar experiments were performed to evaluate miRNA inhibitors of Let-7. TuD Let-7 inhibitors were identified that can de-repress luciferase expression mediated by up to 7 copies of Let-7 sponge sequences (Let-7 binding sites). A 2-fold increase in the expression of Dicer mRNA, a Let-7 target, was also observed. Similarly, TuD Let-7, but not Let-7 sponges, induced Dicer protein levels in HeLa cells. Induction of Dicer gene expression was also observed in mice liver and heart infected with rAAV (Serotype 9) harboring TuD Let-7 inhibitor genes (for up to 7 weeks post infection) with no adverse effects on liver function observed. Administration of rAAV-Let-7-Antag increased by 2-fold the mRNA levels of Dicer, the enzyme that produces miRNAs from pre-miRNAs and which is normally repressed

by Let-7. The results of this study indicate that rAAV-miR-Antags mediate efficient and stable somatic inhibition of miRNAs and will provide both an efficient tool to study miRNA function as well as a potential therapeutic for dyslipidemia, in the case of miR122, and other diseases caused by miRNA deregulation.

Example 2: rAAV- mediated therapeutic silencing of miR-122 leads to rapid and significant reduction of LDL in DLDR$^{-/-}$/Apobec 1$^{-/-}$ mice

MicroRNA (miRNA) regulation was evaluated as an alternative to FH gene therapy. miRNAs play critical roles in regulating most cellular processes. The most abundant miRNA in the liver, miR-122 regulates cholesterol metabolism by an unknown mechanism(s) and does not directly target LDLR mRNA. Recombinant AAV9 was examined for efficient and stable miR-122 antagonism in normal C57BL/6 mice by expressing an optimized miRNA-122 antagonist (Antag). A single intravenous injection of rAAV9-miR-122Antag (SEQ ID NO: 1) produced an 80% decrease in the level of mature miR-122 and 3-fold up-regulation of four miR-122 target genes as well as a 50% reduction in total serum cholesterol, HDL, and LDL in male mice fed a regular diet (Figure 4A, 4B, and 4C). This inhibition was observed for up to 14 weeks post infection with no significant impact on liver function as assessed by Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) gene expression levels. ALT and AST are enzymes located in liver cells that leak out into the general circulation when liver cells are injured. To assess the therapeutic potential of miR-122 inhibition, the same vector was administered to adult male and female LDLR-/-/Apobec 1A-/- mice, the most comparable mouse model of human FH with the normal chow diet (Powell-Braxton L, et al., Nature Medicine, Volume 4, Number 8, August 1998.) One week after dosing, a 20% decrease in total serum cholesterol was observed in both males and females. Interestingly, the decreases in males were exclusively in the HDL fraction, whereas the declines in females were exclusively in LDL. By the second week, total cholesterol and LDL in the treated females had declined about 30% but HDL levels remained unchanged. (See, Figure 5A, 5B, and 5C.) The reduction of total cholesterol in males remained at 20%, reflecting a 50% increase in HDL and a 13% drop in LDL as compared to the mice in week 1. The observed sex-specific differences in miR-122 inhibition may reflect the previously reported lower efficiency of rAAV-mediated liver transduction in female mice, suggesting that doses may be optimized for rAAV-mediated therapeutic inhibition of miR-122 for the treatment of FH (Davidoff AM, et al., Blood. 2003;102:480-488). The results of this study indicate that rAAV can achieve efficient and

stable somatic miRNA inhibition providing basis for a therapy for dyslipidemia and other diseases caused by miRNA deregulation.

Example 3: AAV vector-mediated *in vivo* miRNA antagonism for treating hyperlipidemia

Genetic disruption of a miRNA gene represents a powerful strategy to study miRNA function, but many miRNA genes share the same seed sequence - the 6-8 nt miRNA region that defines its target repertoire - and therefore one member of a miRNA family can compensate for loss of another. Creation of an animal model in which all members of a miRNA family are deleted is daunting. Moreover, humans and mice share more than 276 miRNAs, requiring hundreds of conditional knockout strains to assess the function and contribution to disease of each conserved miRNA in adult mice. Chemically modified anti-miRNA oligonucleotides (AMO) complementary to mature miRNAs are widely available tools for miRNA inhibition *in vitro* and *in vivo*[3-9]. Effective AMOs typically employ expensive or proprietary chemical modifications such as 2′-*O*-methyl, 2′-*O*-methoxyethyl, or 2′,4′-methylene (locked nucleic acid; LNA), and current chemistries and formulations do not permit safe and effective delivery of AMOs to many tissues or organs. Additionally, miRNA inhibition with AMOs requires repeated administrations to suppress expression of the cognate miRNA[3,7-11].

As an alternative to AMOs, plasmid DNA vectors that express miRNA "sponges" - multiple, tandem miRNA binding sites designed to competitively inhibit miRNA function and expressed from an RNA polymerase II promoter - have been used to study miRNA function in cultured cells[12] and *in vivo* in flies[13]. Depletion of miR-223 in hematopoietic cells using a sponge-expressing lentiviral vector to stably modify hematopoietic stem cells *ex vivo*, followed by bone marrow reconstitution in mice, produced a phenotype similar to that observed in a genetic miRNA knockout[14]. However, the risk of insertional mutagenesis and the requirement for *ex vivo* manipulation may limit the use of the lentiviral vector-based miRNA inhibition for functional genomics studies and therapeutic applications. More recently, compact, RNA polymerase III-driven miRNA decoys have been reported, including "Tough Decoy" (TuD) RNAs and miRZips, both of which enable stable and permanent inhibition of miRNA in cultured cells and *in vivo*[16]. Nevertheless, a method to stably and efficiently antagonize miRNAs for studying miRNA-target interactions in adult mammals remains to be developed.

The 4.7 kb single-stranded DNA parvovirus Adeno-associated virus[17] (AAV) is a widespread, nonpathogenic resident in primates, including humans[18,19]. In the past decade, new recombinant AAV (rAAV) vectors have been created from natural AAV serotypes, providing efficient gene transfer vehicles that target diverse tissues in mice and non-human primates[20-23].

Here, the use of rAAV vectors in mice to inhibit miR-122, a miRNA highly abundant in liver[24], and *let*-7, a miRNA with functions in cancer and development[25] is reported. Different promoters (RNA polymerase II versus RNA polymerase III) and designs of miRNA antagonists (sponge, TuD, and miRZip) were evaluated in cultured cells, and RNA polymerase III-driven TuD was identified as the most potent miRNA antagonist. rAAV9 vectors were engineered expressing anti-miR-122 and anti-*let-7* TuD RNAs and were used to achieve efficient, sustained and target-specific miR-122 or *let-7* inhibition *in vivo*. Each miRNA inhibitor increased the expression of the corresponding miRNA target genes in adult mice. High throughput sequencing of liver miRNAs from the treated mice confirmed that the targeted miRNA, but no other miRNAs, were depleted. Moreover, miRNA depletion *in vivo* was accompanied by the 3′ addition of non-templated nucleotides as well as 3′-to-5′ shortening of the miRNA, a degradation pathway previously observed *in vivo* in *Drosophila melanogaster* and *in vitro* in transformed, cultured human cells[33]. Importantly, sustained phenotypic changes were observed in the serum cholesterol profiles of both wild-type C57BL/6 and low density lipoprotein (LDL) receptor-deficient mice treated with rAAV9-expressing the anti-miR-122, but not the anti-*let-7*, TuD RNA. The data provided herein suggest that rAAV-expressing TuD RNAs could enable stable therapy for hypercholesterolemia and other disorders caused by miRNA expression.

Evaluation of transcribed miRNA antagonists in cultured cells

To test different transcribed miRNA antagonists, a highly abundant miRNA, miR-122, which regulates cholesterol biosynthesis in the liver, and an anti-oncogenic miRNA, *let-7*, were chosen as targets for inhibition. A series of miR-122 and *let-7* antagonists were designed including miRNA sponges, TuD RNAs (**Figure 6**) and miRZips[12,15] (www.systembio.com/microrna-research/microrna-knockdown/mirzip/) (**Table 2**). miRNA sponges were expressed using the RNA polymerase II simian vacuolating virus (SV40) promoter, or the liver-specific, human thyroid hormone-binding globulin (TBG) promoter, or, alternatively, the RNA polymerase III U6 promoter; the U6 promoter was used to drive TuD

and miRZip expression (**Fig. 7a and b**).

Oligonucleotide	Sequence (5′ to 3′)
anti-miR-122 TuD	GGATCCGACGGCGCTAGGATCATCAACCAAACACCATT GATCTTCACACTCCACAAGTATTCTGGTCACAGAATACA ACCAAACACCATTGATCTTCACACTCCACAAGATGATC CTAGCGCCGTCTTTTTTGAATTC (SEQ ID NO: 19)
anti-*let-7* TuD	GGATCCGACGGCGCTAGGATCATCAACAACTATACAAC CATCTTACTACCTCACAAGTATTCTGGTCACAGAATACA ACAACTATACAACCATCTTACTACCTCACAAGATGATCC TAGCGCCGTCTTTTTTGAATTC (SEQ ID NO: 20)
miR-122 miRZip	GGATCCTGGTCAGTGACAATGTTTGCTTCCTGTCAGACA AACACCATTGTCACACTCCATTTTTAAGCTTGAAGACAA TAGC (SEQ ID NO: 21)
anti-*let-7* miRZip	GGATCCTCTCGTAGTAGGTTGTATAGTTCTTCCTGTCAG AAACTATACAACCTACTACCTCATTTTTAAGCTTGAAGA CAATAGC (SEQ ID NO: 22)
anti-miR-122 sponge	TCTAGACAAACACCATACAACACTCCACAAACACCATA CAACACTCCACAAACACCATACAACACTCCACAAACAC CATACAACACTCCACAAACACCATACAACACTCCACAA ACACCATACAACACTCCACAAACACCATACAACACTCC AGGGCCC (SEQ ID NO: 23)
anti-*let-7* sponge	TCTAGAAACTATACAAAACCTACCTCAAACCACACAAA ACCTACCTCAAACCATACAAAACCTACCTCAAACTATG CAAAACCTACCTCTAACTATACAAAACCTACCTCAAAC TGTACAAAACCTACCTCAAACCATACAAAACCTACCTC AGCCCTAGA (SEQ ID NO: 24)
Mutant anti-miR-122 sponge	TCTAGACAAACACCATACAACAAGAAACAAACACCATA CAACAAGAAACAAACACCATACAACAAGAAACAAACA CCATACAACAAGAAACAAACACCATACAACAAGAAAC AAACACCATACAACAAGAAACAAACACCATACAACAA GAAAGGGCCC (SEQ ID NO: 25)
Mutant anti-*let-7* sponge	TCTAGAAACTATACAAAACCTAAAGAAAACCACACAAA ACCTAAAGAAAACCATACAAAACCTAAAGAAAACTATG CAAAACCTAAAGATAACTATACAAAACCTAAAGAAAAC TGTACAAAACCTAAAGAAAACCATACAAAACCTAAAGA AGGGCCC (SEQ ID NO: 26)
(miR-122)1 sense	pCGAAACAAACACCATTGTCACACTCCATT (SEQ ID NO: 27)
(miR-122)1 anti-sense	pCGAATGGAGTGTGACAATGGTGTTTGTTT (SEQ ID NO: 28)
(miR-122)3 sense	pCGAAACAAACACCATTGTCACACTCCAACAAACACCA TTGTCACACTCCAA CAAACACCATTGTCACACTCCATT (SEQ ID NO: 29)
(miR-122)3 anti-sense	pCGAATGGAGTGTGACAATGGTGTTTGTTGGAGTGTGAC AATGGTGTTTGTT GGAGTGTGACAATGGTGTTTGTTT (SEQ ID NO: 30)

XbaI–ApaI linker F	CTAGATTCCGAGATATCGGTAATGGGCC (SEQ ID NO: 31)
XbaI–ApaI linker R	GGCCCATTACCGATATCTCGGAATCTAG (SEQ ID NO: 32)
pri-miR-122 F	ATCGGGCCCGACTGCAGTTTCAGCGTTTG (SEQ ID NO: 33)
pri-miR-122 R	CGCGGGCCCGACTTTACATTACACACAAT (SEQ ID NO: 34)
Nras F	TGGACACAGCTGGACAAGAG (SEQ ID NO: 35)
Nras R	CTGTCCTTGTTGGCAAGTCA (SEQ ID NO: 36)
Kras F	CAAGAGCGCCTTGACGATACA (SEQ ID NO: 37)
Kras R	CCAAGAGACAGGTTTCTCCATC (SEQ ID NO: 38)
Hras1 F	CGTGAGATTCGGCAGCATAAA (SEQ ID NO: 39)
Hras1 R	GACAGCACACATTTGCAGCTC (SEQ ID NO: 40)
Mm-Dicer F	GCAGGCTTTTTACACACGCCT (SEQ ID NO: 41)
Mm-Dicer R	GGGTCTTCATAAAGGTGCTT (SEQ ID NO: 42)
c-MYC F	CAACGTCTTGGAACGTCAGA (SEQ ID NO: 43)
c-MYC R	TCGTCTGCTTGAATGGACAG (SEQ ID NO: 44)
Hfe2 F	GGGGACCTTGCTTTCCACTC (SEQ ID NO: 45)
Hfe2 R	GCCTCATAGTCACAGGGATCT (SEQ ID NO: 46)
Tmed3 F	AGCAGGGCGTGAAGTTCTC (SEQ ID NO: 47)
Tmed3 R	TTGTACGTGAAGCTGTCATACTG (SEQ ID NO: 48)
Aldolase A F	TGGGAAGAAGGAGAACCTGA (SEQ ID NO: 49)
Aldolase A R	AGTGTTGATGGAGCAGCCTT (SEQ ID NO: 50)
CAT-1 F	TACCAGTGGCCGTGTTTGTA (SEQ ID NO: 51)
CAT-1 R	GCTGTTGCCAAGCTTCTACC (SEQ ID NO: 52)
Cyclin G1 F	AATGGCCTCAGAATGACTGC (SEQ ID NO: 53)
Cyclin G1 R	AGTCGCTTTCACAGCCAAAT (SEQ ID NO: 54)
Mm-Actin F	ATGCCAACACAGTGCTGTCTGG (SEQ ID NO: 55)
Mm-Actin R	TGCTTGCTGATCCACATCTGCT (SEQ ID NO: 56)
miR-122 probe	TGGAGTGTGACAATGGTGTTTG (SEQ ID NO: 57)
Let-7 probe	AACTATACAACCTACTACCTCA (SEQ ID NO: 58)
miR-26a probe	AGCCTATCCTGGATTACTTGAA (SEQ ID NO: 59)
miR-22 Probe	ACAGTTCTTCAACTGGCAGCTT (SEQ ID NO: 60)

U6 probe	CTCTGTATCGTTCCAATTTTAGTATA (SEQ ID NO: 61)
IDT miRNA cloning linker-1	AppCTGTAGGCACCATCAAT/ddC/ (SEQ ID NO: 62)
5′ Illumina RNA Adapter	GUUCAGAGUUCUACAGUCCGACGAUC (SEQ ID NO: 63)
Small RNA RT primer	ATTGATGGTGCCTACAG (SEQ ID NO: 64)
Small RNA PCR Primer 1	CAAGCAGAAGACGGCATACGAATTGATGGTGCCTACAG (SEQ ID NO: 65)
Small RNA PCR Primer2	AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACA GTCCGA (SEQ ID NO: 66)

Table 2.

To evaluate the efficiency of each miRNA antagonist, the ability of the expression constructs to de-repress a nuclear-targeted *E. coli β-galactosidase (nLacZ)* reporter mRNA containing 1 or 3 copies of fully complementary miR-122-binding sites in the 3′ untranslated region (UTR) was tested. The *nLacZ* reporter plasmid was co-transfected with the various miR-122 inhibitor constructs or a control plasmid into HuH-7 cells[27], a human hepatoma cell line expressing ~16,000 miR-122 molecules per cell[27]. As expected, reporter expression was reduced ~50% when one miR-122-binding site was present in the *nLacZ* 3′ UTR and >80% when three sites were present (**Fig. 7c**). Among the RNA polymerase II-driven anti-miR-122 sponges, only the TBG promoter, a strong liver-specific promoter, detectably increased expression of *nLacZ* bearing a single miR-122binding site, indicating that the sponge partially inhibited miR-122. However, nLacZ expression was not significantly increased by this sponge when the reporter contained three miR-122-binding sites (**Fig. 7c**), suggesting that the change in miR-122 activity or concentration was too small to overcome the greater repression conferred by three miRNA target sites.

In contrast, both the one- and three-site reporters were de-repressed by the RNA polymerase III-driven anti-miR-122 TuD RNA. For the one-site reporter, the TuD restored *nLacZ* expression to that observed when no miR-122 target sites were present in the reporter (**Fig. 7c**). The greater efficacy of the TuD RNA might reflect the higher level of transcription possible with RNA polymerase III compared to RNA polymerase II, greater miRNA inhibition by the TuD design, or both. To distinguish among these possibilities, the ability of three different U6-driven miR-122 antagonist constructs - sponge, TuD, and miRZip - to de-repress the *nLacZ* reporter containing three miR-122-binding sites was compared. Again, only the TuD significantly (p-value ≤ 0.001) derepressed nLacZ repression by miR-122 in HuH-7 cells (**Fig. 7d**). The anti-miR-122 TuD expression construct was similarly effective in

human embryonic kidney (HEK) 293 cells. Because HEK 293 cells express little miR-122, pri-miR-122 was expressed from a plasmid, which was co-transfected along with the *nLacZ* reporter with or without three miR-122-binding sites and the TuD-expressing plasmid. *nLacZ* expression was scored 48 h later. The anti-miR-122 TuD, but not an anti-*let-7* TuD or an anti-miR-122 or anti-*let-7* sponge, significantly derepressed reporter expression in the presence of the miR-122 expression plasmid (**Fig. 7e**).

miRNAs that are extensively complementary to their targets direct Argonaute2 protein to cleave the mRNA, whereas less extensive complementarity generally decreases mRNA stability. To test if the TuD RNA can also inhibit repression directed by a miRNA with imperfect complementarity to its target, a firefly luciferase (*Fluc*) reporter mRNA was designed with seven copies of a bulged miR-122-binding site in its 3′ UTR; *Fluc* with seven7 mutant sites served as a control. The miR-122-responsive *Fluc* reporter, anti-miR-122, anti-*let-7* or control TuD plasmid, and, as an internal control, a *Renilla reniformis* luciferase (*Rluc*) expression plasmid, were introduced into HuH-7 cells by transfection. The anti-miR-122 TuD, but not the control or anti-*let-7* TuDs, fully de-repressed Fluc expression (**Fig. 7f**). it is concluded that TuD RNAs are potent and specific miRNA inhibitors.

Finally, the anti-*let-7* TuD increased expression of both the Dicer mRNA and protein; *dicer* is an endogenous *let-7* target[28,29] (**Fig. 8a, 8b,** and **Fig. 9**). Together, the *in vitro* data suggest that the TuD RNA transcribed from a U6 promoter was the most potent of the miRNA antagonists surveyed.

Real-Time monitoring of specific endogenous miRNA activities in live animals

To test the ability of TuD RNAs to inhibit miRNA function *in vivo*, a series of rAAV vector genomes expressing a miRNA-responsive *Gaussia* luciferase (Gluc)[30] mRNA was constructed (**Fig. 10a**). Gluc is a secreted protein, enabling detection of the reporter in the blood or urine of live animals. Seven bulged miR-122 or *let-7* target sites were inserted into the 3′ UTR of the *Gluc* mRNA to render it miRNA responsive. A U6 promoter-driven expression cassette for either an anti-miR-122 or an anti-*let-7* TuD RNA was inserted into the intron of the *Gluc* transcription unit. Reporter lacking either the seven miRNA-binding sites or the TuD expression cassette or both served as controls. miR-122 comprises 70% of total miRNAs in liver[27], posing a stringent test for the ability of TuD RNAs to inhibit the function of even the most abundant miRNA species. *In vitro* in HuH-7 cells, the anti-miR-122, but not anti-*let-7*, TuD RNA derepressed the Gluc reporter bearing seven miR-122-binding sites (**Fig**

10b). Similarly, in HeLa cells anti-*let-7* TuD RNA derepressed the Gluc reporter bearing seven *let-7*binding sites (**Fig 10c**).

Both miR-122 and *let-7* are present in liver[27], and *let-7* is also found in heart[31]. The rAAV genomes were packaged with the AAV9 capsid, which preferentially transduces liver and heart. To further improve transduction, all rAAVs were prepared as self-complementary (sc) genomes[32]. The vectors were administered intravenously to adult male C57B/6 mice and Gluc activity was monitored in blood. Initially, Gluc activity was comparable among the animals injected with vectors expressing the miR-122-regulated reporters, irrespective of the presence of a TuD RNA expression cassette (days 3 and 7). By week 2, Gluc activity declined in the mice that received vectors lacking the antimiR-122 TuD, while Gluc activity increased in the mice treated with the anti-miR-122 TuD expressing vector (**Fig. 10d**). Similarly, Gluc activity was low in mice that received the *let-7*-regulated reporter and was high in mice that received the same reporter containing the anti-let-7 TuD expression cassette. One notable difference between the miR-122- and *let-7*-regulated Gluc reporters was that the *let-7*-regulated reporter was silenced at the earliest time point (day 3), whereas the miR-122-regulated reporter showed an initial lag in achieving silencing (**Fig. 10d.e**). De-repression of Gluc expression by either anti-miR-122 or anti-*let-7* TuD RNA was sustained for the duration of the study, 18 weeks (**Fig. 10d, e**).

scAAV9-delivered TuD RNAs mediate specific miRNA depletion in mouse liver

Four weeks after the administration of scAAV9 vectors, miRNA expression was analyzed in the liver using quantitative RT-PCR. An ~80% reduction in miR-122 was observed in the mice that received the anti-miR-122 TuD expressing vector, compared to vector expressing anti-*let-7* TuD or control vector lacking a TuD (**Fig. 11a**). Northern blot analysis confirmed the reduction of miR-122 in the mice that received anti-miR-122 TuD (**Fig. 11b** and **Fig. 12**). *let-7* was similarly reduced in the mice treated with scAAV9 vectors expressing the anti-*let-7* TuD (the *let-7* Northern probe employed cannot distinguish among the eight mouse *let-7* isoforms). In contrast, no reduction was detected for miR-26a or miR-22, two other abundant liver miRNAs (**Fig. 11b** and **Fig. 12**).

High throughput sequencing of miRNAs from the treated livers further supports the view that scAAV9-delivered TuD RNAs effectively and specifically trigger the destruction of complementary miRNAs. The TuD targeting miR-122 (**Fig. 7b**) reduced the abundance of full-length, 23 nt miR-122 by 4.3-fold (**Fig. 11c**), consistent with the qRT-PCR results (**Fig.**

11a). The 21 and 22 nt miR-122 isoforms decreased less, whereas 20 and 19 nt isoforms increased, suggesting that the TuD triggered 3′-to-5′ exonucleolytic trimming of miR-122 (**Fig. 11c**). Like antagomir-directed destruction of miRNAs in human cell culture[33] the anti-miR-122 TuD promoted the addition of nontemplated nucleotides to the 3′ end of miR-122 (**Fig. 11d**). Prefix-matching reads— sequences that initially match the mouse genome but then end with non-templated nucleotides—doubled in the mouse expressing the anti-miR-122 TuD, compared to the control (**Fig. 11d**). The 3′ non-templated nucleotides comprised one or more adenosines. Even in the absence of the TuD, 30% of miR-122 was tailed with adenosine, suggesting that miR-122 undergoes post-transcriptional modification, perhaps as part of its natural turnover.

Mouse liver expresses all eight *let-7* isoforms (**Fig. 13**). These isoforms differ by 1–4 nucleotides outside their common seed sequence (**Fig. 11e**). Anti*let-7* TuD strongly decreased the abundance of those full-length *let-7* isoforms that were fully complementary to the TuD sequence (*let-7a*, 12.1-fold) or contained only a single non-seed mismatch to the TuD (*let-7c*, 5.1-fold; *let-7d*, 5.0-fold; and *let-7f*, 11.0-fold). In contrast, the decrease was smaller for *let-7b* (1.6-fold) and *let-7g* (2.7-fold), which contain two 3′ mismatches to the TuD, *let-7i* (1.5-fold), which contains three 3′ mismatches to the TuD, and *let-7e* (3.6-fold), which contains a purine:purine mismatch to the TuD at position 9, immediately flanking the seed sequence (**Fig. 11e, 11f**). Prefix-matching reads increased more for *let-7a*, *c*, *d*, and *f* - the *let-7* isoforms that decreased the most in response to the anti-*let-7* TuD - whereas *let-7 b*, *e*, *g* and *i*, which decreased least showed no increase in such trimmed-and-tailed species (**Fig. 11g**). These findings indicate that anti-*let-7* TuD-directed miRNA decay requires nearly perfect complementarity between TuD-RNA and the miRNA. For both the anti-miR-122 and the anit-*let-7* TuDs, the overall abundance of other miRNAs was unaltered (**Fig. 14**).

scAAV9-delivered anti-miRNA TuD RNAs specifically increase expression of endogenous miRNA-regulated mRNAs

When delivered using scAAV9, anti-miRNA TuD RNAs also de-repress miR-122- and *let-7*-regulated endogenous mRNAs (**Fig. 15**). qRT-PCR was used to analyze the expression of validated targets of miR-122 and *let-7* in liver and heart four weeks after injection of the TuD-expressing scAAV9 vectors. Mice injected with scAAV9 expressing the Gluc reporter but with no TuD RNA served as a control. For mice treated with the vector expressing anti-miR-122 TuD RNA, a 2.5 to 3.5-fold increase in *Aldolase A* (3.3 ± 0.5; p-value ≤ 0.04),

Tmed3 (4.2 ± 1.5; *p*-value ≤ 0.01), *Hfe2* (3.3 ± 1.0; *p*-value ≤ 0.02), and *Cyclin G1* (2.5 ± 0.4; *p*-value ≤ 0.001) mRNAs[7,34] was detected in the liver, four genes previously shown to be regulated by miR-122; expression of these four mRNAs was unaltered in the heart, which lacks miR-122 (**Fig. 15**). No statistically significant change in the expression of the four miR-122-regulated mRNAs was found in either liver or heart from mice that received the vector expressing anti-*let-7* TuD RNA (**Fig. 15**).

The miRNA-producing enzyme Dicer[29] itself is repressed by *let-7* family miRNAs. qRT-PCR was used to measure *Dicer* mRNA abundance in mice that received scAAV9 vector expressing either anti-miR-122 or anti-*let-7* TuD RNA (**Fig. 15**). When *let-7* was inhibited, *Dicer* mRNA was increased in both liver (1.9 ± 0.2; *p*-value ≤ 0.001) and heart (2.4 ± 0.4; *p*-value ≤ 0.003). The RAS family genes, HRAS, NRAS and KRAS, have been reported also to be repressed by the *let-7* miRNA[35-37]. Increased expression of *Nras* was observed in both liver (1.3 ± 0.1; *p*-value ≤ 0.01) and heart (1.3 ± 0.1; *p*-value ≤ 0.02) and of *Hras1* (1.3 ± 0.1; *p*-value ≤ 0.04) in heart in the mice that received scAAV9 expressing the anti-*let-7*, but not the anti-miR-122 TuD RNA, relative to the control (**Fig. 15**).

Anti-miR-122 TuD RNA reduces cholesterol levels

miR-122 is required for normal cholesterol biosynthesis; inhibition of miR-122 with AMOs decreases cholesterol metabolism in adult mice[7,9,11] and non-human primates[8,10]. In wild-type mice, a single intravenous injection of scAAV9 expressing anti-miR-122 RNA significantly reduced total serum cholesterol (45 ± 5%; *p*-value ≤ 0.001) and high-density lipoprotein (HDL, 42 ± 5%; *p*-value ≤ 0.001) levels beginning two weeks after injection, and this reduction was sustained for the 18 week duration of the study. LDL levels were also reduced (88 ± 102%; *p*-value ≤ 0.05) by the third week and lasted for the duration of the study (**Fig. 16a**). Total serum cholesterol, HDL, and LDL levels were unaltered in mice that received the anti-*let-7* TuD. The body weight and liver function of the mice were normal throughout the study: no weight loss (**Fig. 17**) or statistically significant increase in serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels was detected (**Fig. 16b**).

High cholesterol is a major risk factor for cardiovascular disease, the most common cause of morbidity and mortality in the United States. Mutations in the LDL receptor (*LDLR*) gene cause the common inherited dyslipidemia, familial hypercholesterolemia[38]. rAAV-mediated replacement of the LDL receptor represents a promising approach for the treatment

of this genetic disorder, but may be limited by host immunity against the therapeutic gene product[39,40]. Sustained miR-122 inhibition could provide an alternative therapy for familial hypercholesterolemia. scAAV9-delivered anti-miR-122 TuD RNA was evaluated as potential treatment for familial hypercholesterolemia using a mouse model of the human disease: $LDLR^{-/-}$, Apobec $1A^{-/-}$ double mutant mice fed a normal chow diet[41]. One month after a single intravenous dose of scAAV9 expressing anti-miR-122 TuD RNA, total serum cholesterol was reduced by 34 ± 3% (p-value ≤ 0.0006), serum HDL decreased 18 ± 2% (p-value ≤ 0.02), and serum LDL, which is the therapeutic target for familial hypercholesterolemia in female mice, decreased 53 ± 6% (p-value ≤ 0.006), compared to mice that received the scrambled TuD control (**Fig. 16c**). In male mice, a 21 ± 1% (p-value ≤ 0.05) reduction in total cholesterol, a 26 ± 2% (p-value ≤ 0.004) reduction in HDL, and a 20 ± 1% (p-value ≤ 0.02) reduction in LDL was measured (**Fig. 16c**). The observed sex specific differences in lowering cholesterol in the $LDLR^{-/-}$, Apobec $1A^{-/-}$ mice warrant further investigation.

DISCUSSION

The large number of mammalian miRNAs makes identifying their biological functions a daunting challenge. Inhibitors of miRNA function promise to accelerate the understanding of miRNA biology, especially in adult mammals. Strategies to inhibit miRNAs include complementary chemically modified oligonucleotides and transcribed miRNA-binding competitor RNAs. While effective miRNA inhibitors, chemically modified oligonucleotides are currently expensive, some modifications are not commercially available, and require repeated dosing that risks long-term toxicity. Moreover, many tissues are not currently accessible to delivery of oligonucleotides.

Transcribed miRNA-binding RNAs provide an alternative to oligonucleotides. The small size of their transcripts makes them readily incorporated into a variety of gene transfer vectors. Primate AAV-derived vectors represent attractive tools for this application because of their unique tissue tropism, high efficiency of transduction, stability of *in vivo* gene transfer, and low toxicity[22,42].

Recently, several designs of miRNA antagonists - sponges, TuD RNAs, and miRZips - have been developed and tested in lentiviral vectors *in vitro*[12,15] and in genetic knockout animal models *in vivo*[13,14,16]. These miRNA antagonists were compared *in vitro*, and the most effective design, the TuD RNAs, was used *in vivo* to inhibit miR-122 and *let-7* by incorporating TuD expression cassettes into scAVV9. The data provided herein demonstrate

that a single administration of rAAV9 expressing a TuD RNA provides a stable and efficient reduction in the level of the targeted miRNA (**Fig. 11**), leading to an increase in expression of its endogenous target mRNAs (**Fig. 8** and **15**), and a corresponding phenotypic change in metabolism (**Fig. 16**). The high throughput sequencing data provided herein suggest that, in mice, TuD RNAs inhibit their miRNA targets via the same target-RNA directed tailing and trimming pathway as recently described in flies for engineered[33] and endogenous mRNAs[43] and for synthetic oligonucleotide "antagomirs" in cultured human HeLa cells[33] (**Fig. 11**). The data presented here, which are the first observations of target RNA-directed miRNA tailing, trimming, and destruction in a living mammal, suggest that this pathway may be widely conserved among animals.

To date, methods to monitor miRNA function in live adult mammals have not been described. The *in vivo* Gluc sensor system described here provides a simple means to detect changes in specific miRNA function, such as those caused by miRNA inhibitors (**Fig. 10**). This system allows one to assess the activity of a specific miRNA *in vitro* in a cell line or *in vivo* in a tissue or organ, providing a quantitative measure of the effectiveness of a miRNA antagonist in live animals across time.

Retrospective profiling has linked aberrant miRNA expression to a variety of diseases, suggesting that miRNAs may provide new targets for therapy[44-48]. Indeed, miR-122 inhibition by AMOs[7-11] or scAAV-delivered TuD RNA (**Fig. 16**) lowers both HDL and LDL. However, the current view that HDL protects against heart attack[49] argues that therapy for dyslipidemia should lower LDL but raise HDL levels. Recently, miR-33 was identified as a repressor of HDL biogenesis; miR-33 inhibition raises serum HDL level[16]. Perhaps simultaneous inhibition of miR-122 and miR-33 by a pair of TuD RNAs expressed from a single scAAV vector may achieve a more balanced and healthy cholesterol profile and provide long-lasting therapy for familial hypercholesterolemia.

Low miR-122 levels have been associated with hepatocellular carcinoma in rodents and humans[50-52], although no direct causal link has been established[51,52]. Because AAV vector expression is stable for years in rodent and primate models, animals treated with scAAV9 expressing anti-miR-122 should enable testing the safety of prolonged miR-122 inhibition in general and the increased risk of developing hepatocellular carcinoma in particular.

Materials and Methods

Construction of miRNA antagonist and sensor plasmids

siFluc fragment in pRNA-U6.1/Neo-siFluc (GenScript, Piscataway, NJ) was replaced with TuD miR-122, TuD *let-7*, miR-122Zip, *let-7*Zip, miR-122 sponge *and let-7* sponge that were designed as previously described[12,15] (http://www.systembio.com/microrna-research/microrna-knockdown/mirzip/) to generate U6-driven expression cassettes for expression of different miRNA antagonists. The design of *let-7* antagonist was based on the consensus sequence of all *let-7* family members. The XbaI-ApaI linker was generated by annealing oligonucleotide pairs, XbaI-ApaI linker F and XbaI-ApaI linker R (**Table 2**) followed by cloning into the ApaI site after Fluc gene in pGL3-control plasmid. The chemically synthesized miR-122 or *let-7* sponge sequence flanked with XbaI and ApaI sites was digested and cloned into pGL3-XbaI-ApaI linker plasmid to create SV40 promoter-driven sponge expression cassettes. Then, the fragment containing Fluc gene and miR-122 or *let-7* sponge was isolated by NcoI and ApaI double digestions from pGL3 miR-122 sponge or pGL3 *let-7* sponge and cloned into the KpnI site of pAAVCBPI vector plasmid or between PstI and MluI sites of pAAVTBGPI vector plasmid to generate CB promoter and TBG promoter driven sponge expression vectors, respectively.

One or three copies of perfectly complementary miRNA target sites were designed based on the annotated miRNA sequences in miRBase[53] and inserted into the BstBI restriction site in the 3′ UTR of the *nLacZ* expression cassette of the ubiquitously-expressed pAAVCB *nuclear-targeted β-galactosidase (nLacZ)* plasmid using synthetic oligonucleotides (**Table 2**). To express miR-122, pri-miR-122 fragment was amplified by PCR from mouse genomic DNA with specific oligonucleotides (**Table 2**), cloned into the XbaI restriction site right after a firefly luciferase cDNA in the pAAVCB *Fluc* plasmid. The identity of pri-miR-122 was verified by sequencing. scAAV9 vectors used in this study were generated, purified, and titered as previously described[18].

To create AAV vectors, seven copies of bulged target sites for miR-122 or *let-7* were synthesized and cloned into BclI site after the Gluc reporter gene in the pscAAVCBPI Gluc plasmid. The EcoRI and HindIII fragment harboring U6-TuDmiR-122 or U6-TuD *let-7* expression cassette was isolated from pRNA-U6.1/Neo-TuDmiR-122 or pRNA-U6.1/Neo-TuD *let-7* plasmid and cloned into PpuMI site in the intron region of pscAAVCBPI Gluc with or without bulged target sites for miR-122 or *let-7*.

Cell culture

HEK 293, HuH-7 and HeLa cells were cultured in Dulbecco's Modified Eagle

Medium supplemented with 10% FBS and 100 mg/L of penicillin-streptomycin (HyClone, South Logan, UT). Cells were maintained in a humidified incubator at 37°C and 5% CO2. Plasmids were transiently transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions.

Luciferase reporter assay

Cells were lysed with passive lysis buffer (Dual-Glo Luciferase Assay System, Promega, Madison, WI) and 10 µl of lysis was used for the assay. Firefly and *Renilla* luciferase activities were assessed using the Dual-Glo Luciferase Assay System (Promega, Madison, WI) in accordance with the manufacturer's instructions. The *Gaussia* luciferase (Gluc) assay was performed following the procedure described previously[30]. Briefly, 10 µl each of culture media from the indicated transfections was used for the *in vitro* Gluc assay. To monitor Gluc expression *in vivo*, the study animals were bled from a superficial cut on facial vein made by a 5.5 mm animal lancet (MEDIpoint, Mineola, NJ) at different time points after AAV9 vector treatment. Five µl each of blood samples was used for the Gluc assay.

Mice

C57BL/6 mice (Charles River Laboratories) and LDLR$^{-/-}$/Apobec 1A$^{-/-}$ mice (Dr. James Wilson, University of Pennsylvania) were maintained and used for the study according to the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Four-to-six weeks old wild type C57BL/6 male mice were treated with AAV vectors at 1×10^{12} genome copies/mouse or 5×10^{13} genome copies/kg by tail vein injection. To evaluate therapeutic potential of scAAV9TuDmiR122, 4 to 6 weeks old LDLR$^{-/-}$/Apobec 1A$^{-/-}$ mice were treated with TuD-miR-122 or Scrambled vector at a dose of 3×10^{11} genome copies/mouse or 1.5×10^{13} genome copies/kg by tail vein injection. To monitor lipid profiles of the study animals, the serum samples were collected at different times after AAV9 vector injection and analyzed for total cholesterol, HDL and LDL on a COBAS C 111 analyzer (Roche Diagnostics, Lewes, UK). For RNA analyses, the animals were necropsied at 4 weeks after the treatment; liver and heart tissues were harvested for RNA preparation.

qRT-PCR analysis

RNA was extracted using Trizol (Invitrogen Carlsbad, CA), according to the manufacturer's instructions. Total RNA (0.5–1 μg) was primed with random hexamers and reverse-transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). Quantitative PCR reactions were performed in triplicate with 0.3 μM gene specific primer pairs (**Table 2**) using the GoTaq qPCR master mix (Promega, Madison, WI) in a StepOne Plus Real-time PCR device (Applied Biosystems, Foster City, CA). The expression of mature miR-122 and U6 was assayed using the TaqMan microRNA Assay (Applied Biosystems, Foster City, CA).

Northern blot analysis

To detect miR-122, miR-26a, miR-22 and *let-7* in total liver RNA, 10 μg of total RNA was resolved by denaturing 15% polyacrylamide gels, transferred to Hybond N+ membrane (Amersham BioSciences, Pittsburgh, PA), and cross-linked with 254 nm light (Stratagene, La Jolla, CA). Synthetic DNA oligonucleotides (**Table 2**), 5′ end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase (NEB, Beverly, MA), were used as probes for miR-122, miR-26a, miR-22 and *let-7* and U6 (**Table 2**) and hybridized in Church buffer (0.5 M NaHPO4, pH 7.2, 1 mM EDTA, 7% [w/v] SDS) at 37°C. Membranes were washed using 1× SSC, 0.1% (w/v) SDS buffer, and then visualized using a FLA-5100 Imager (FUJIFILM, Tokyo, Japan).

Small RNA sequencing

Small RNA libraries were constructed and sequenced as described[33]. Briefly, 50 μg total RNA was isolated with the mirVana kit (Ambion Foster City, CA), 19–29 nt small RNAs were separated and isolated through gel electrophoresis using 15% polyacrylamide/urea gel (SequaGel, National Diagnostics, Atlanta, GA). IDT miRNA cloning linker-1 was ligated to the 3′ of small RNAs using truncated T4 RNA ligase 2 (NEB, Beverly, MA) and gel purified; a 5′ RNA adapter was ligated to the 3′ ligated RNA with T4 RNA ligase (NEB, Beverly, MA). The ligation product was used as template for reverse transcription with Small RNA RT primer. The cDNA was amplified with small RNA PCR primer 1 and RNA PCR primer 2. The PCR product was gel-purified and submitted for high throughput sequencing. For sequencing statistics see **Tables 3** and **4**. Small RNA analyses were as previously described[33]. Sequence data are available through the NCBI Short Read Archive (www.ncbi.nlm.nih.gov/sites/sra) as GSE25971.

Sample	Total reads	Reads perfectly	Reads matching	Small RNA reads	Pre miRNA matching

		matching genome	annotated ncRNAs	(excluding ncRNAs)	reads
Control	3,239,264	2,335,379	8,894	2,326,485	2,174,544
anti-miR-122 TuD	3,386,944	2,189,155	16,366	2,172,789	1,917,478
anti-*let-7* TuD	1,893,012	1,232,744	4,021	1,228,723	1,163,466

Table 3. Sequencing statistics: Analysis of 5′ prefix-matching reads. To detect small RNAs bearing 3′ terminal, non-templated nucleotides, reads matching the reference genome for only part of their entire length were identified.

Sample	Total reads	Prefixes matching genome	Prefixes excluding internal mm	Prefixes matching annotated ncRNAs	Prefixes (excluding ncRNAs)	Pre miRNA matching prefixes
Control	3,239,264	903,885	800,519	1,920	798,599	568,087
Anti-miR-122 TuD	3,386,944	1,197,478	1,086,409	2,855	1,083,554	775,181
Anti-let-7 TuD	1,893,012	660,268	574,454	1,008	573,446	344,092

Table 4.

Western blot analysis

Proteins were extracted with RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40 [v/v], 1% sodium deoxycholate [w/v], 0.1% SDS [w/v]) containing a protease inhibitor mixture (Boston BP). Protein concentration was determined using the Bradford method. Protein samples, 50 µg each, were loaded onto 10% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membrane (Amersham BioSciences,). Immunoblotting was performed using the LI-COR infrared imaging system. Briefly, membranes were blocked with blocking buffer (LI-COR) at room temperature for 2 h, followed by incubation with either anti-GAPDH (Millipore), anti-Dicer (Santa Cruz) for 2 h at room temperature. After three washes with PBS plus 0.1% Tween-20 (v/v), membranes were incubated for 1 h at room temperature using secondary antibodies conjugated to LI-COR IRDye. Signals were detected using the Odyssey Imager (LICOR).

Statistical analysis

All results are given as mean ± standard deviation and compared between groups using the two-tailed Student's t-test, except in **Fig. 16c**, where the *p*-value was calculated using the Mann-Whitney test.

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The entire contents of references 1-53 listed above, and of all other references, publications, or database entries identified herein are incorporated into this application by reference as if each individual reference, publication, or database entry was incorporated herein by reference individually. In case of a conflict, the instant disclosure shall control.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in this description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

Having thus described several aspects of at least one embodiment of this invention, it is to be appreciated various alterations, modifications, and improvements will readily occur to

those skilled in the art. Such alterations, modifications, and improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawings are by way of example only.

CLAIMS

What is claimed is:

1. A method for treating a high cholesterol-related disorder in a subject, the method comprising:

administering an effective amount of a recombinant Adeno-Associated Virus (rAAV) to the subject, wherein the rAAV comprises at least one transgene that expresses a miRNA inhibitor that inhibits the expression of miR-122 in the subject.

2. The method of claim 1, wherein the miRNA inhibitor comprises an miR-122 binding site.

3. The method of claim 1 or 2, wherein the miR-122 binding site is flanked by two stem sequences.

4. The method of any one of claims 1-3, wherein the miR-122 binding site comprises a non-binding, central portion that is not complementary with miR-122, flanked by two portions that are complementary with miR-122.

5. The method of claim 1, wherein the miRNA inhibitor comprises a first miR-122 binding site and a second miR-122 binding site, wherein a first stem sequence flanks the first miR-122 binding site at its 5'-end, a second stem sequence flanks the first miR-122 binding site at its 3'-end and the second miR-122 binding site at its 5'-end, and a third stem sequence flanks the second miR-122 binding site at its 3'-end.

6. The method of claim 5, wherein each of the two miR-122 inhibitor binding sites comprises a non-binding, central portion that is not complementary with miR-122.

7. The method of claim 6, wherein the non-binding, central portion of the first miR-122 binding site is at least partially complementary with the non-binding, central portion of the second miR-122 binding site.

8. The method of claim 6 or 7, wherein the non-binding, central portion of the first miR-122 binding site is complementary with the non-binding, central portion of the second miR-122 binding site at 1 to 5 nucleotides.

9. The method of any one of claims 6-8, wherein the non-binding, central portion of the first miR-122 binding site is complementary with the non-binding, central portion of the second miR-122 binding site at 3 nucleotides.

10. The method of any one of claims 6-9, wherein the non-binding, central portion of the first miR-122 binding site has a length in a range of 1 to 10 nucleotides.

11. The method of any one of claims 6-10, wherein the non-binding, central portion of the first miR-122 binding site has a length in a range of 3 to 5 nucleotides.

12. The method of any one of claims 6-11, wherein the non-binding, central portion of the first miR-122 binding site has a length of 4 nucleotides.

13. The method of any one of claims 6-12, wherein the non-binding, central portion of the second miR-122 binding site has a length in a range of 1 to 10 nucleotides.

14. The method of any one of claims 6-13, wherein the non-binding, central portion of the second miR-122 binding site has a length in a range of 3 to 5 nucleotides.

15. The method of any one of claims 6-14, wherein the non-binding, central portion of the second miR-122 binding site has a length of 4 nucleotides.

16. The method of any one of claims 6-15, wherein the first miR-122 binding and the second miR-122 binding site are complementary at a sequence of 2 to 10 nucleotides in length.

17. The method of any one of claims 6-16, wherein the first miR-122 binding and the second miR-122 binding site are complementary at a sequence of 4 nucleotides in length.

18. The method of any one of claims 1-17, wherein the miRNA inhibitor comprises two or more miR-122 binding sites.

19. The method of any one of claims 1-18, wherein the miRNA inhibitor comprises or consists of a sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 21, or SEQ ID NO: 23.

20. The method of any one of claims 1-19, wherein the rAAV has a capsid of the AAV9 serotype, which has a sequence as set forth in SEQ ID NO: 3.

21. The method of any one of claims 1-20, wherein the rAAV has a capsid that is a variant of the capsid of the AAV9 serotype.

22. The method of claim 21, wherein the rAAV has a capsid of the AAV9 serotype variant, Csp-3, which has a sequence as set forth in SEQ ID NO: 4.

23. The method of any one of claims 1-22, wherein the rAAV targets liver tissue.

24. The method of any one of claims 1-23, wherein the rAAV transduces hepatocytes.

25. The method of any one of claims 1-24, wherein the effective amount of rAAV is 10^{10}, 10^{11}, 10^{12}, or 10^{13} genome copies.

26. The method of any one of claims 1-25, wherein administering is performed intravenously.

27. The method of any one of claims 1-26, wherein administering is performed by injection into the hepatic portal vein.

28. The method of any one of claims 1-27, wherein the subject is a mouse, a rat, a rabbit, a dog, a cat, a sheep, a pig, or a non-human primate.

29. The method of any one of claims 1-28, wherein the subject is a human.

30. The method of any one of claims 1-29, wherein the subject is an animal model of a high cholesterol-related disorder.

31. The method of any one of claims 1-30, wherein the high cholesterol-related disorder is Type I, Type IIa, Type IIb, Type III, Type IV, or Type V Hyperlipoproteinemia.

32. The method of any one of claims 1-31, wherein the high cholesterol-related disorder is associated with diabetes mellitus, metabolic syndrome, kidney disease (nephrotic syndrome), hypothyroidism, Cushing's syndrome, anorexia nervosa, sleep deprivation, Zieve's syndrome, antiretroviral drugs, diet, high body weight, or low physical activity.

33. The method of any one of claims 1-32, wherein the subject is a human and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 200 mg/dl.

34. The method of any one of claims 1-33, wherein the subject is a mouse and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 100 mg/dl.

35. The method of any one of claims 1-34, wherein the subject is a rat and the high cholesterol-related disorder is characterized by total serum cholesterol level greater than or equal to 70 mg/dl.

36. A nucleic acid vector comprising:

(a) a first promoter operably linked with a transgene that comprises:

(i.) a protein coding region, and

(ii.) at least one binding site of a test miRNA; and

(b) a second promoter operably linked with a miRNA inhibitor coding region, wherein the miRNA inhibitor specifically binds to the test miRNA.

37. The nucleic acid vector of claim 36, wherein the first promoter is a RNA Polymerase II promoter.

38. The nucleic acid vector of claim 36 or 37, wherein the second promoter is a RNA Polymerase III promoter.

39. The nucleic acid vector of any of claims 36-38 further comprising a first untranslated region between the first promoter and at least a portion of the protein coding region, wherein the second promoter and the miRNA inhibitor coding region are positioned within the first untranslated region.

40. The nucleic acid vector of claim 39, wherein the first untranslated region is positioned at the 5' end of the complete protein coding region.

41. The nucleic acid vector of claim 39, wherein the first untranslated region is positioned within an intron of the protein coding region.

42. The nucleic acid vector of any of claims 36-41, wherein the transgene further comprises a second untranslated region, wherein the at least one binding site of the test miRNA is in the second untranslated region.

43. The nucleic acid vector of claim 42, wherein the second untranslated region is positioned at the 3' end of the complete protein coding region.

44. The nucleic acid vector of any of claims 36-43 further comprising a pair of inverted terminal repeats that flank the first promoter and the transgene.

45. The nucleic acid vector of claim 44, wherein the pair of inverted terminal repeats further flank the second promoter and the miRNA inhibitor coding region.

46. The nucleic acid vector of any of claims 36-45, wherein the protein coding region encodes a reporter protein selected from: a fluorescent protein, luciferase, β-

galactosidase, secreted alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase (CAT), and β-lactamase.

47. A method for assessing the effectiveness of a miRNA inhibitor, the method comprising:

(a) transfecting a cell with a nucleic acid vector of any one of claims 36 to 46,

wherein the miRNA inhibitor coding region encodes the miRNA inhibitor; and

(b) determining the level of expression of the protein encoded by the protein coding region in the cell, wherein the level of expression of the protein is indicative of the effectiveness of the miRNA inhibitor.

48. The method of claim 47 further comprising contacting the cell with the test miRNA.

49. The method of claim 47 or 48 wherein the cell expresses the test miRNA.

50. The method of any one of claims 36-48, wherein the test miRNA is a mammalian miRNA.

51. The method of any one of claims 36-50, wherein the test miRNA is a human miRNA.

52. The method of any one of claims 36-51, wherein the miRNA inhibitor is a miRNA sponge, an antisense oligonucleotide, or a tough decoy RNA.

53. A method for assessing the effectiveness of a miRNA inhibitor, the method comprising:

(a) transfecting a first cell with a nucleic acid vector of any one of claims 36 to 46,

wherein the miRNA inhibitor coding region encodes the miRNA inhibitor;

(b) transfecting a second cell with the nucleic acid vector,

wherein levels of the test miRNA are lower in the second cell compared with the first cell; and

(c) comparing the level of expression of the protein encoded by the protein coding region in the first cell with the level of expression of the protein encoded by the protein coding region in the second cell,

wherein the results of the comparison in (c) are indicative of the effectiveness of the miRNA inhibitor.

54. A method for assessing the effectiveness of a miRNA inhibitor, the method comprising:

(a) transfecting a cell with a nucleic acid vector of any one of claims 36 to 46,

wherein the miRNA inhibitor coding region encodes the miRNA inhibitor;

(b) determining a first level of expression of the protein encoded by the protein coding region in the cell;

(c) contacting the cell with the test miRNA;

(d) determining a second level of expression of the protein encoded by the protein coding region in the cell; and

(e) comparing the first level of expression of the protein with the second level of expression,

wherein the results of the comparison in (e) are indicative of the effectiveness of the miRNA inhibitor.

55. A kit for assessing the function of a miRNA inhibitor, the kit comprising:

a container housing a nucleic acid vector of any one of claims 36 to 46.

56. A kit for assessing the function of a miRNA inhibitor, the kit comprising:

a container housing a component of a molecular sensing system.

57. A miRNA inhibitor comprising:

a first miRNA binding site and a second miRNA binding site, wherein a first stem sequence flanks the first miRNA binding site at its 5'-end, a second stem sequence flanks the first miRNA binding site at its 3'-end and the second miRNA binding site at its 5'-end, and a third stem sequence flanks the second miRNA binding site at its 3'-end, wherein at least one of the miRNA binding sites comprises a nucleotide sequence complementary to a consensus sequence of the plurality of target miRNAs.

58. The miRNA inhibitor of claim 57, wherein each of the two miRNA inhibitor binding sites comprises a non-binding, central portion that is not complementary with any of the plurality of miRNAs targeted by the miRNA inhibitor.

59. The miRNA inhibitor of claim 58, wherein the non-binding, central portion of the first miRNA binding site is at least partially complementary with the non-binding, central portion of the second miRNA binding site.

60. The miRNA inhibitor of claim 58 or 59, wherein the non-binding, central portion of the first miRNA binding site is complementary with the non-binding, central portion of the second miRNA binding site at 1 to 5 nucleotides.

61. The miRNA inhibitor of any one of claims 58-60, wherein the non-binding, central portion of the first miRNA binding site is complementary with the non-binding, central portion of the second miRNA binding site at 3 nucleotides.

62. The miRNA inhibitor of any one of claims 58-61, wherein the non-binding, central portion of the first miRNA binding site has a length in a range of 1 to 10 nucleotides.

63. The miRNA inhibitor of any one of claims 58-62, wherein the non-binding, central portion of the first miRNA binding site has a length in a range of 3 to 5 nucleotides.

64. The miRNA inhibitor of any one of claims 58-63, wherein the non-binding, central portion of the first miRNA binding site has a length of 4 nucleotides.

65. The miRNA inhibitor of any one of claims 58-64, wherein the non-binding, central portion of the second miRNA binding site has a length in a range of 1 to 10 nucleotides.

66. The miRNA inhibitor of any one of claims 58-65, wherein the non-binding, central portion of the second miRNA binding site has a length in a range of 3 to 5 nucleotides.

67. The miRNA inhibitor of any one of claims 58-66, wherein the non-binding, central portion of the second miRNA binding site has a length of 4 nucleotides.

68. The miRNA inhibitor of any one of claims 58-67, wherein the first miRNA binding site and the second miRNA binding site are complementary at a sequence of 2 to 10 nucleotides in length.

69. The miRNA inhibitor of any one of claims 58-68, wherein the first miRNA binding site and the second miRNA binding site are complementary at a sequence of 4 nucleotides in length.

70. The miRNA inhibitor of any one of claims 57-69, wherein the miRNA inhibitor comprises two or more miRNA binding sites.

71. The miRNA inhibitor of any one of claims 57-70, wherein the miRNA inhibitor is a TuD.

72. The miRNA inhibitor of any one of claims 57 -71, wherein the miRNA inhibitor targets a plurality of let-7 family members.

73. The miRNA inhibitor of claim 71 or 72, wherein the miRNA inhibitor comprises a sequence of at least 8 contiguous nucleotides of SEQ ID NO: 18.

74. The miRNA inhibitor of claim 73, wherein the miRNA inhibitor comprises the first ten nucleotides and/or the last ten nucleotides of SEQ ID NO: 18.

75. The miRNA inhibitor of any of claims 57-71, wherein the miRNA inhibitor comprises or consists of the nucleotide sequence of SEQ ID NO: 18 or SEQ ID NO: 20.

76. A method, comprising

obtaining a consensus sequence from a plurality of miRNAs, and

generating a miRNA inhibitor targeting a plurality of miRNAs, wherein the miRNA inhibitor comprises a first miRNA binding site and a second miRNA binding site,

wherein a first stem sequence flanks the first miRNA binding site at its 5'-end, a second stem sequence flanks the first miRNA binding site at its 3'-end and the second miRNA binding site at its 5'-end, and a third stem sequence flanks the second miRNA binding site at its 3'-end, wherein the miRNA inhibitor comprises a nucleotide sequence complementary to the consensus sequence of the plurality of target miRNAs.

77. The method of claim 76, wherein the consensus sequence is between 5 and 40 nucleotides long.

78. The method of claim 76 or 77, wherein the miRNA is a TuD.

79. A miRNA inhibitor comprising a nucleotide sequence of SEQ ID NO: 22, or SEQ IDNO: 24.

80. A method comprising contacting a cell with an miRNA inhibitor of any one of claims 57 to 75 and 79.

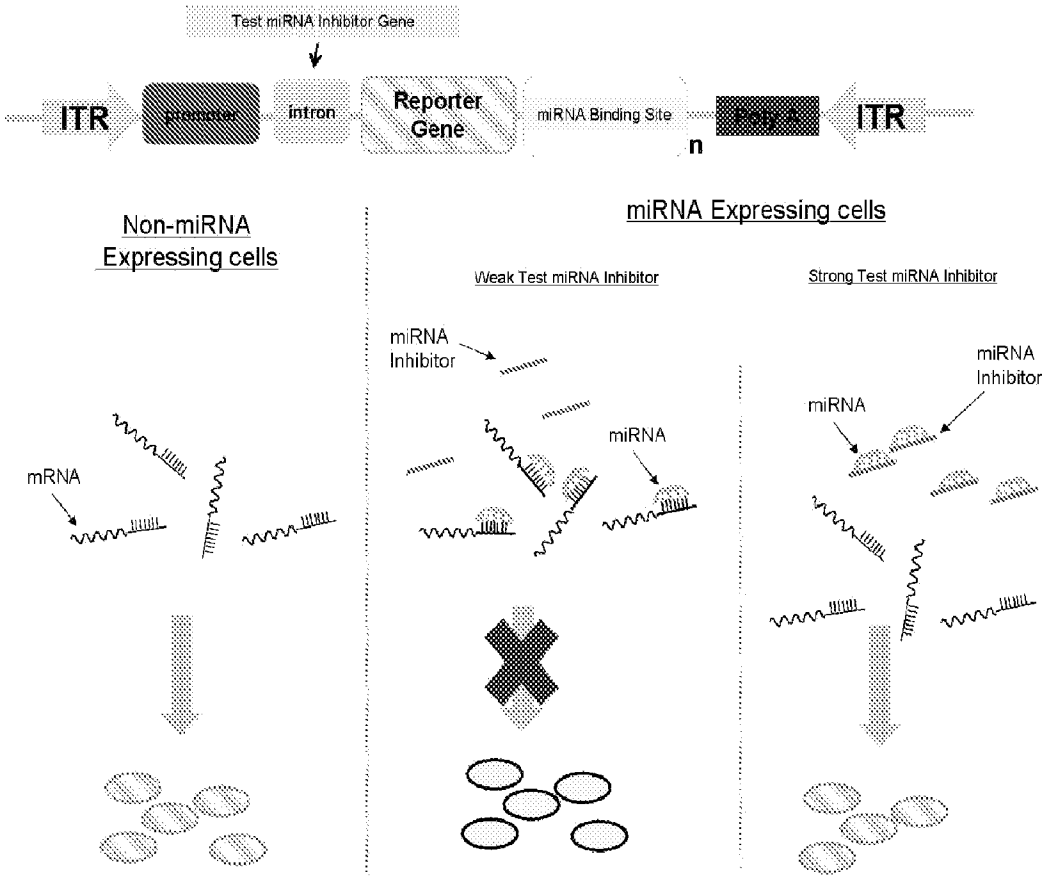
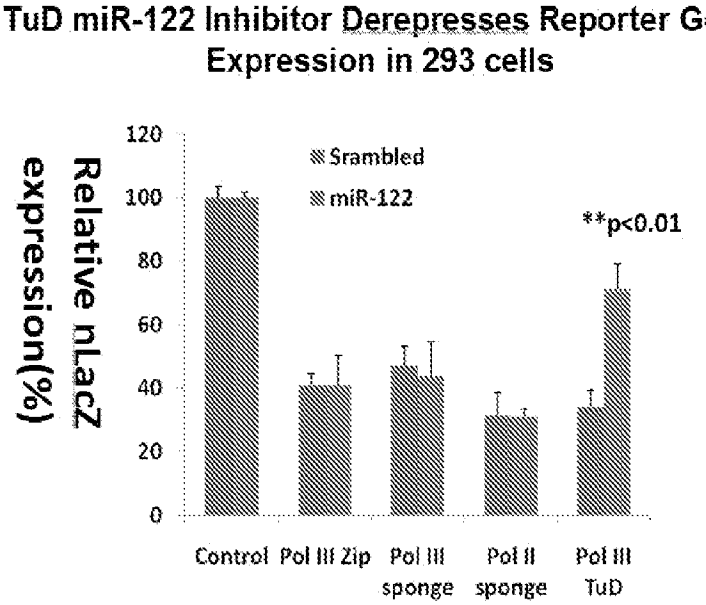
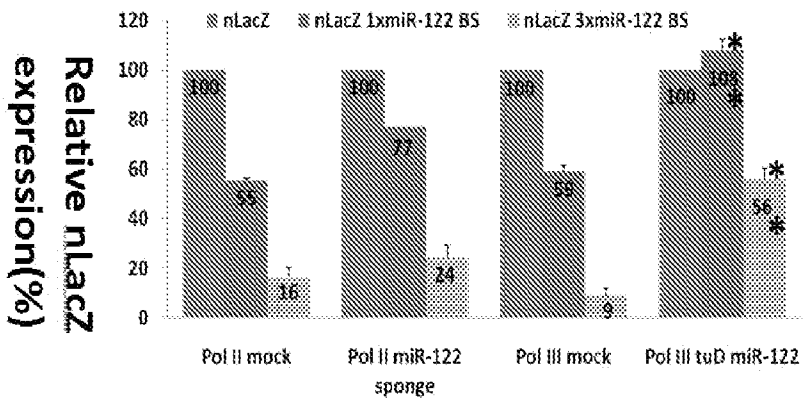

Test miRNA Inhibitor Gene
ITR
promoter
intron
Reporter Gene
miRNA Binding Site
n
Poly A
ITR
Non-miRNA Expressing cells
miRNA Expressing cells
Weak Test miRNA Inhibitor
Strong Test miRNA Inhibitor
mRNA
miRNA Inhibitor
miRNA
miRNA Inhibitor
miRNA

FIGURE 1

A.


TuD miR-122 Inhibitor Derepresses Reporter Gene Expression in 293 cells
Relative nLacZ expression(%)
120
100
80
60
40
20
0
Srambled
miR-122
**p<0.01
Control
Pol III Zip
Pol III sponge
Pol II sponge
Pol III TuD

B.


TuD miR-122 Inhibitor Derepresses Reporter Gene Expression in Huh-7 cells
Relative nLacZ expression(%)
120
100
80
60
40
20
0
nLacZ
nLacZ 1xmiR-122 BS
nLacZ 3xmiR-122 BS
100
55
16
100
77
24
100
59
9
100
103
56
Pol II mock
Pol II miR-122 sponge
Pol III mock
Pol III tuD miR-122

C.

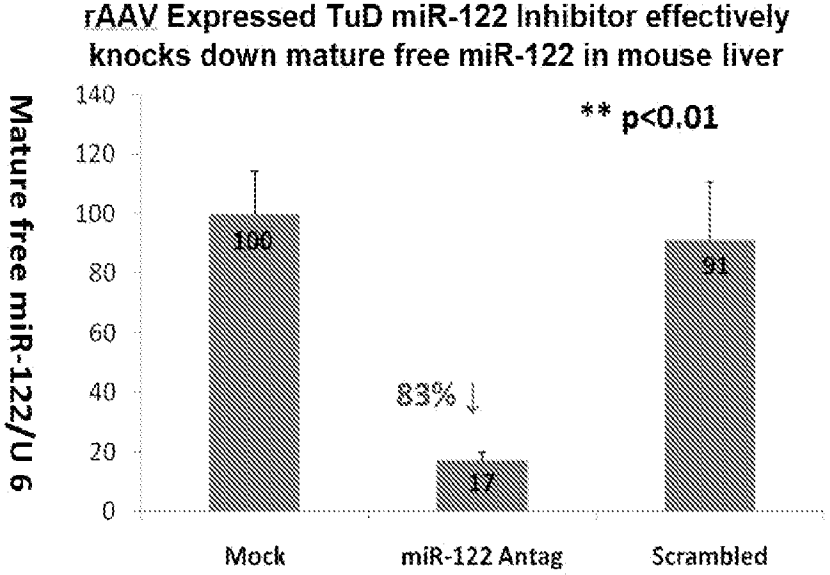

rAAV Expressed TuD miR-122 Inhibitor effectively knocks down mature free miR-122 in mouse liver
Mature free miR-122/U 6
140
120
100
80
60
40
20
0
** p<0.01
100
83% ↓
17
91
Mock
miR-122 Antag
Scrambled

FIGURE 2

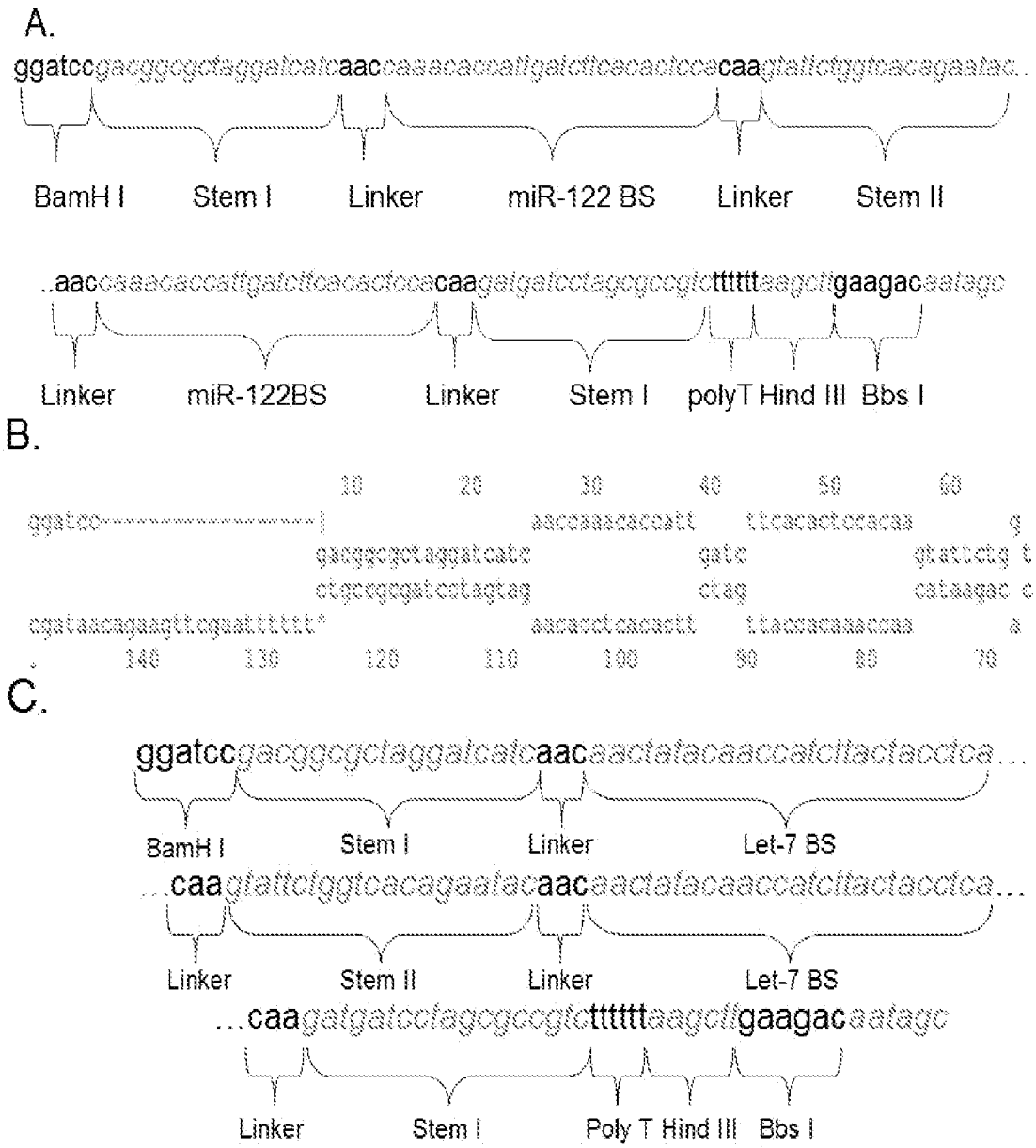

A.
ggatccgacggcgctaggatcatcaaccaaacaccattgatcttcacactccacaagtattctggtcacagaatac..
BamH I
Stem I
Linker
miR-122 BS
Linker
Stem II
..aacccaaacaccattgatcttcacactccacaagatgatcctagcgcgctctttttaagcttgaagacaatagc
Linker
miR-122BS
Linker
Stem I
polyT Hind III
Bbs I
B.
C.
ggatccgacggcgctaggatcatcaacaactatacaaccatcttactacctca...
BamH I
Stem I
Linker
Let-7 BS
...caagtattctggtcacagaatacaacaactatacaaccatcttactacctca...
Linker
Stem II
Linker
Let-7 BS
...caagatgatcctagcgcgctctttttaagcttgaagacaatagc
Linker
Stem I
Poly T Hind III
Bbs I

FIGURE 3

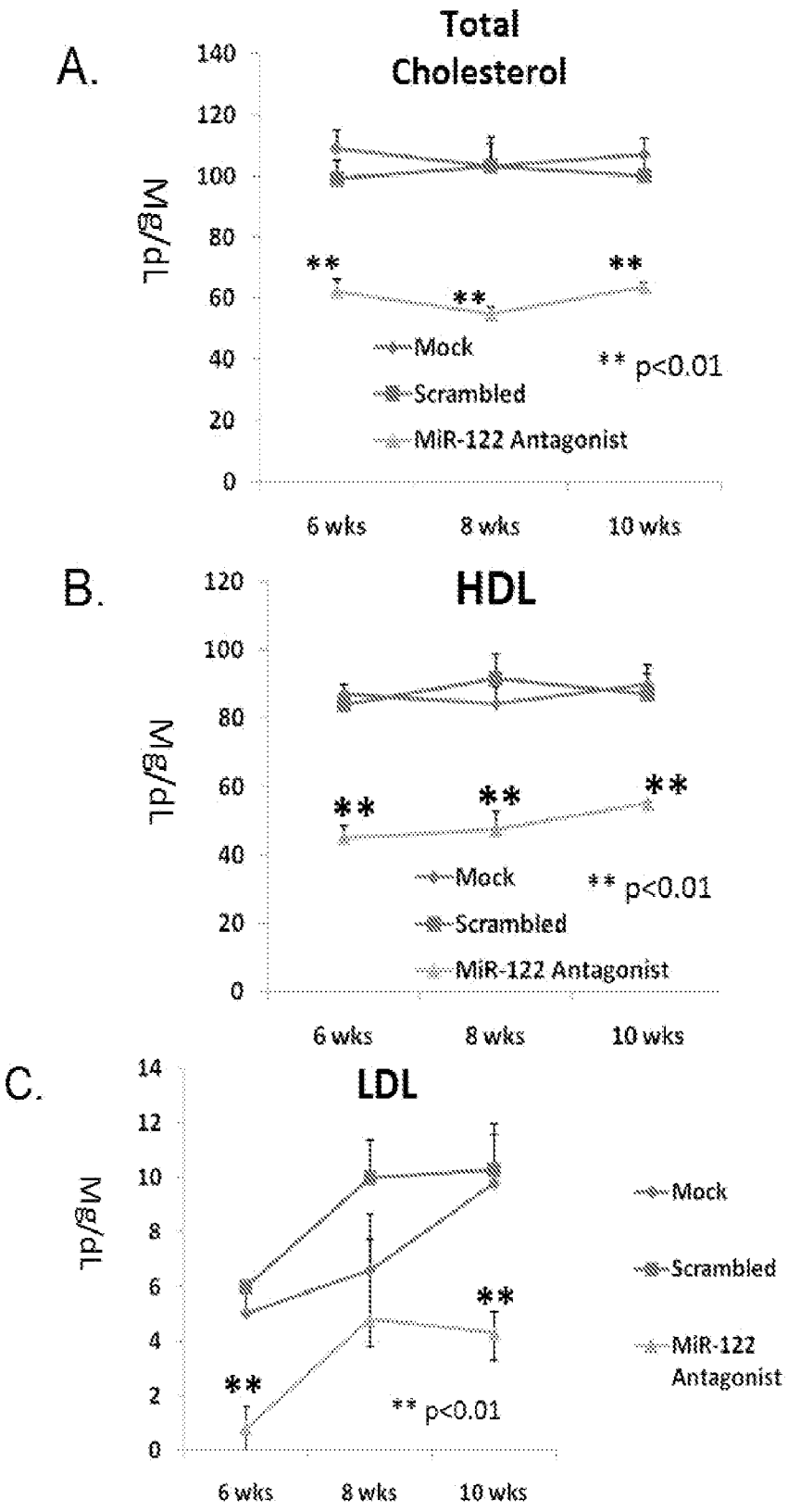

A.
Total Cholesterol
mg/dL
140
120
100
80
60
40
20
0
**
**
**
Mock
Scrambled
MiR-122 Antagonist
** p<0.01
6 wks
8 wks
10 wks
B.
HDL
mg/dL
120
100
80
60
40
20
0
**
**
**
Mock
Scrambled
MiR-122 Antagonist
** p<0.01
6 wks
8 wks
10 wks
C.
LDL
mg/dL
14
12
10
8
6
4
2
0
**
**
** p<0.01
Mock
Scrambled
MiR-122 Antagonist
6 wks
8 wks
10 wks

FIGURE 4

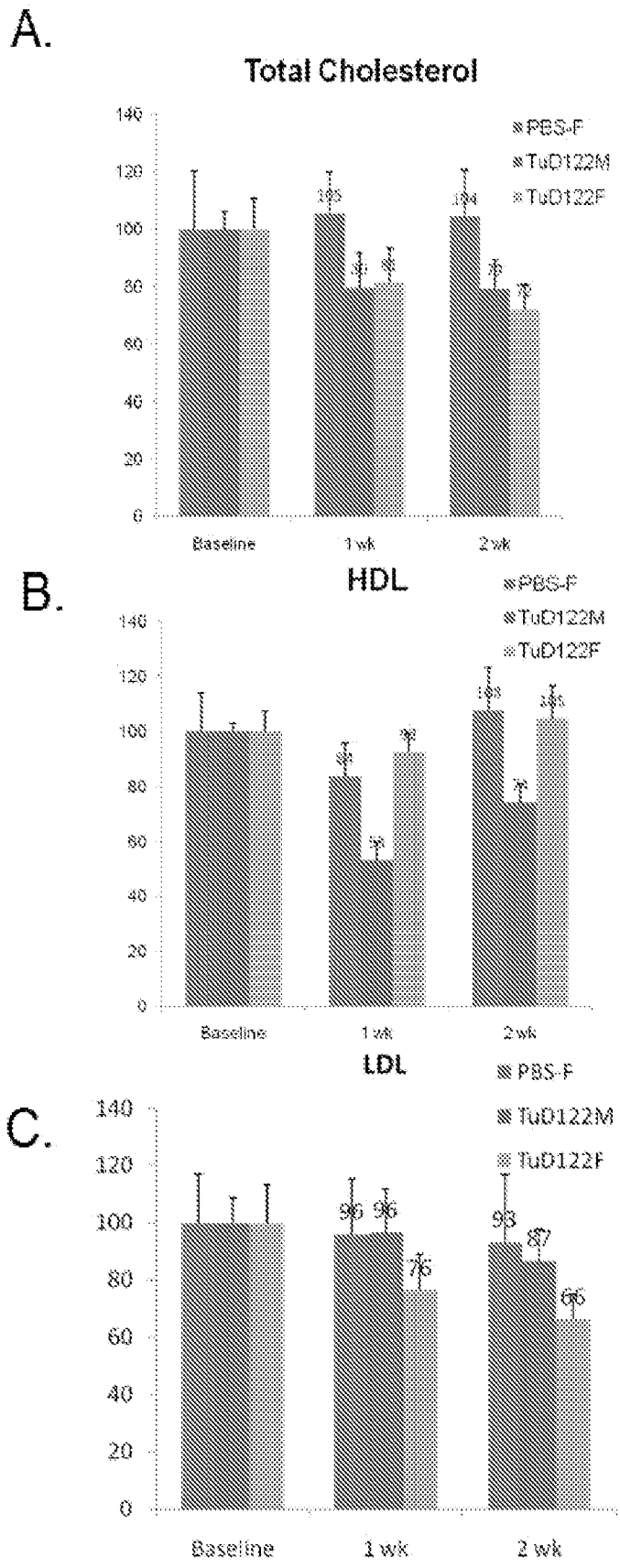

A.
Total Cholesterol
PBS-F
TuD122M
TuD122F
140
120
100
80
60
40
20
0
Baseline
1 wk
2 wk
B.
HDL
PBS-F
TuD122M
TuD122F
C.
LDL
PBS-F
TuD122M
TuD122F
96
96
76
93
87
66

FIGURE 5

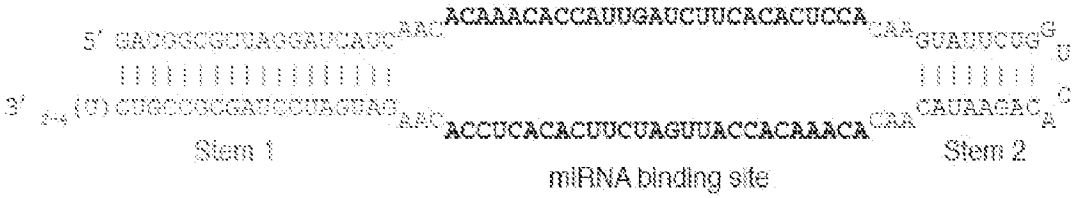


FIGURE 6

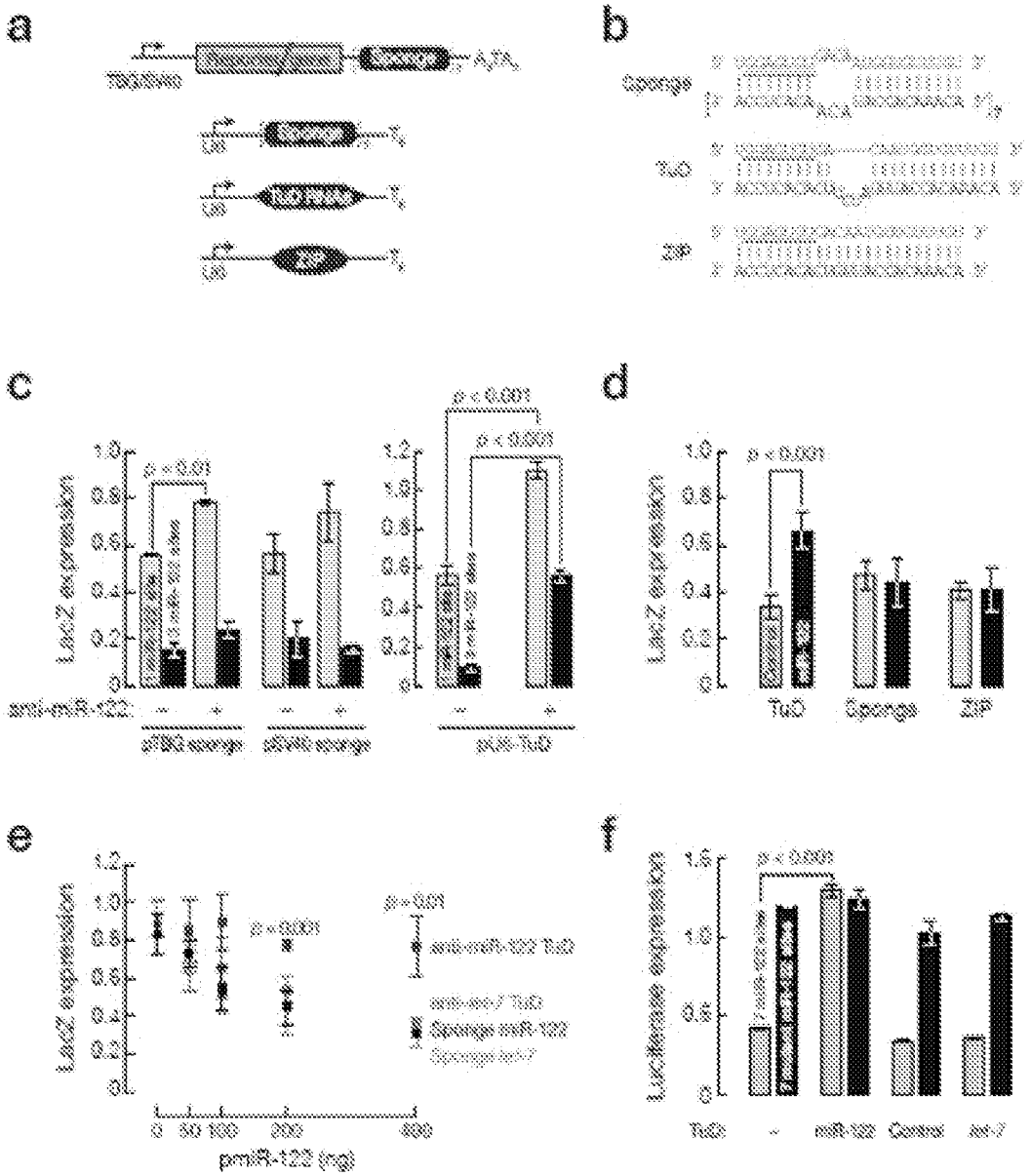


FIGURE 7

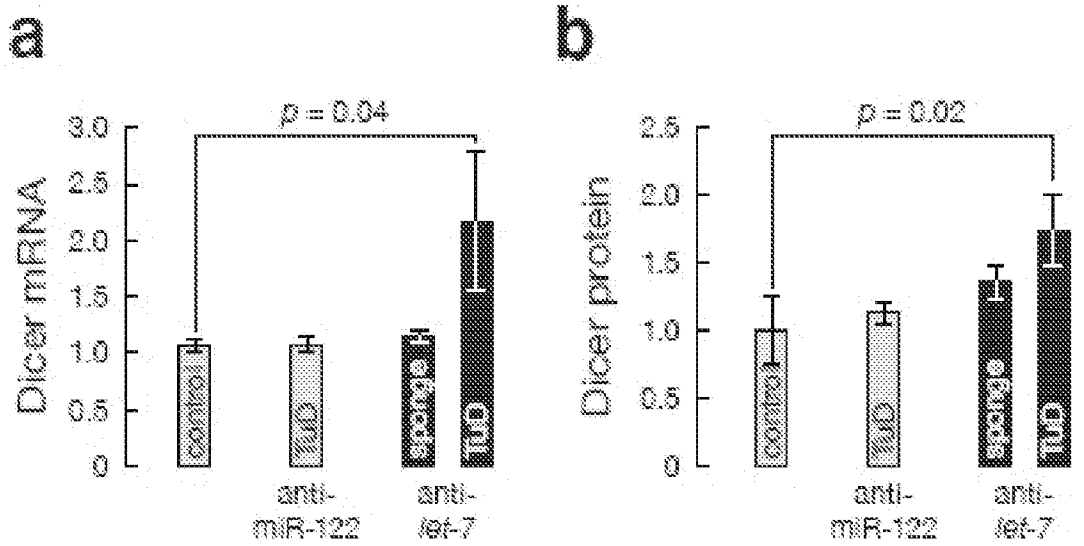


FIGURE 8

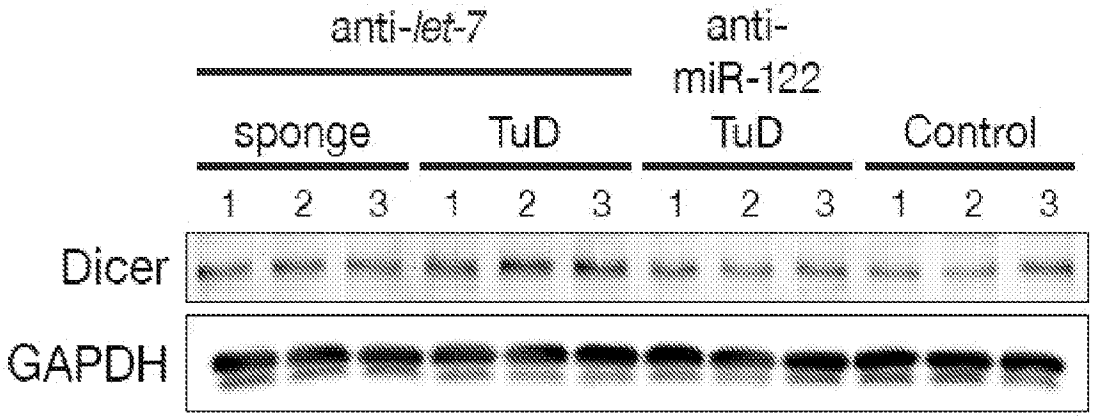


FIGURE 9

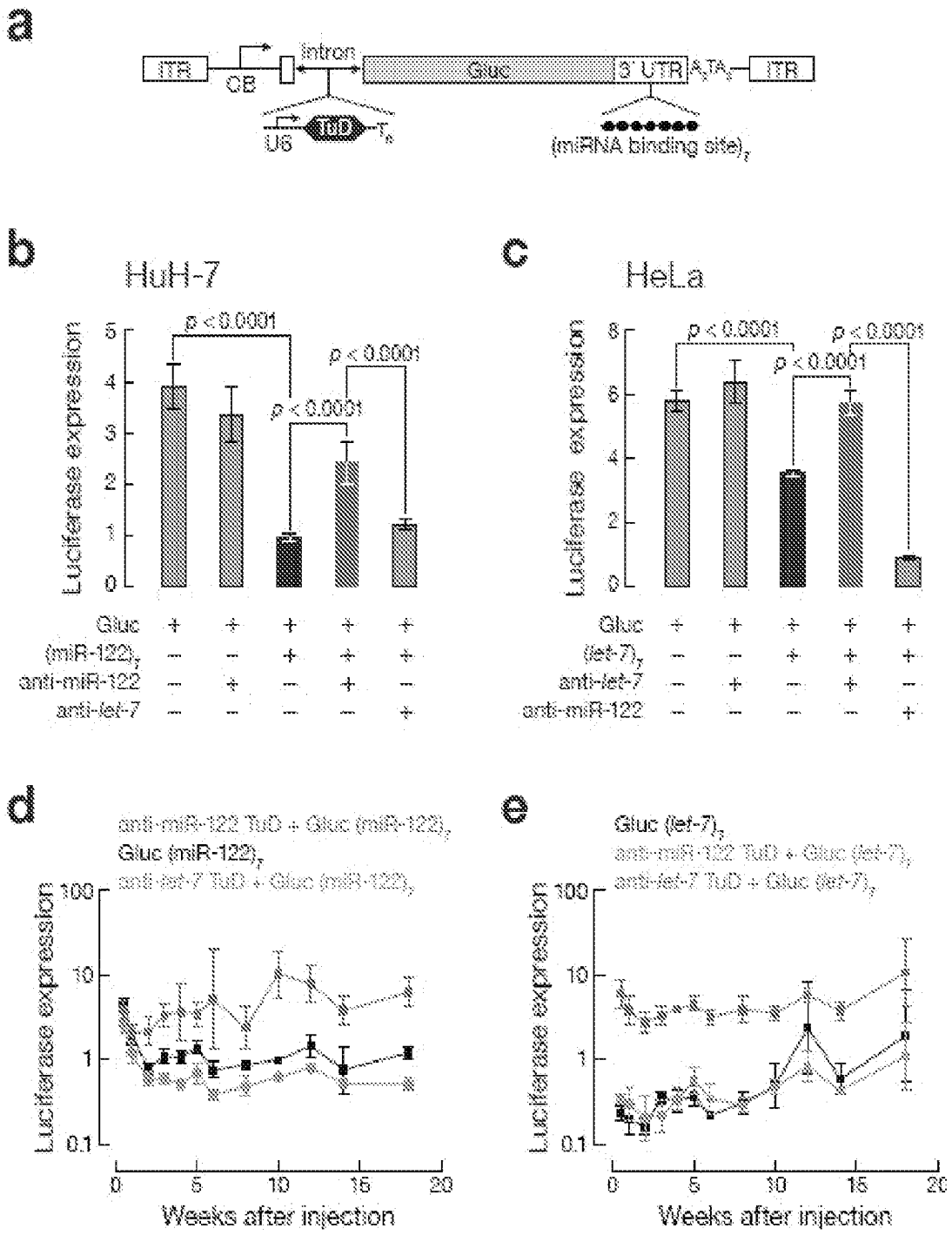

a
ITR
CB
Intron
Gluc
3' UTR
ITR
U6
TuD
(miRNA binding site)
b
HuH-7
Luciferase expression
p < 0.0001
p < 0.0001
p < 0.0001
Gluc
(miR-122)
anti-miR-122
anti-let-7
c
HeLa
Luciferase expression
p < 0.0001
p < 0.0001
p < 0.0001
Gluc
(let-7)
anti-let-7
anti-miR-122
d
anti-miR-122 TuD + Gluc (miR-122)
Gluc (miR-122)
anti-let-7 TuD + Gluc (miR-122)
Luciferase expression
Weeks after injection
e
Gluc (let-7)
anti-miR-122 TuD + Gluc (let-7)
anti-let-7 TuD + Gluc (let-7)
Luciferase expression
Weeks after injection

FIGURE 10

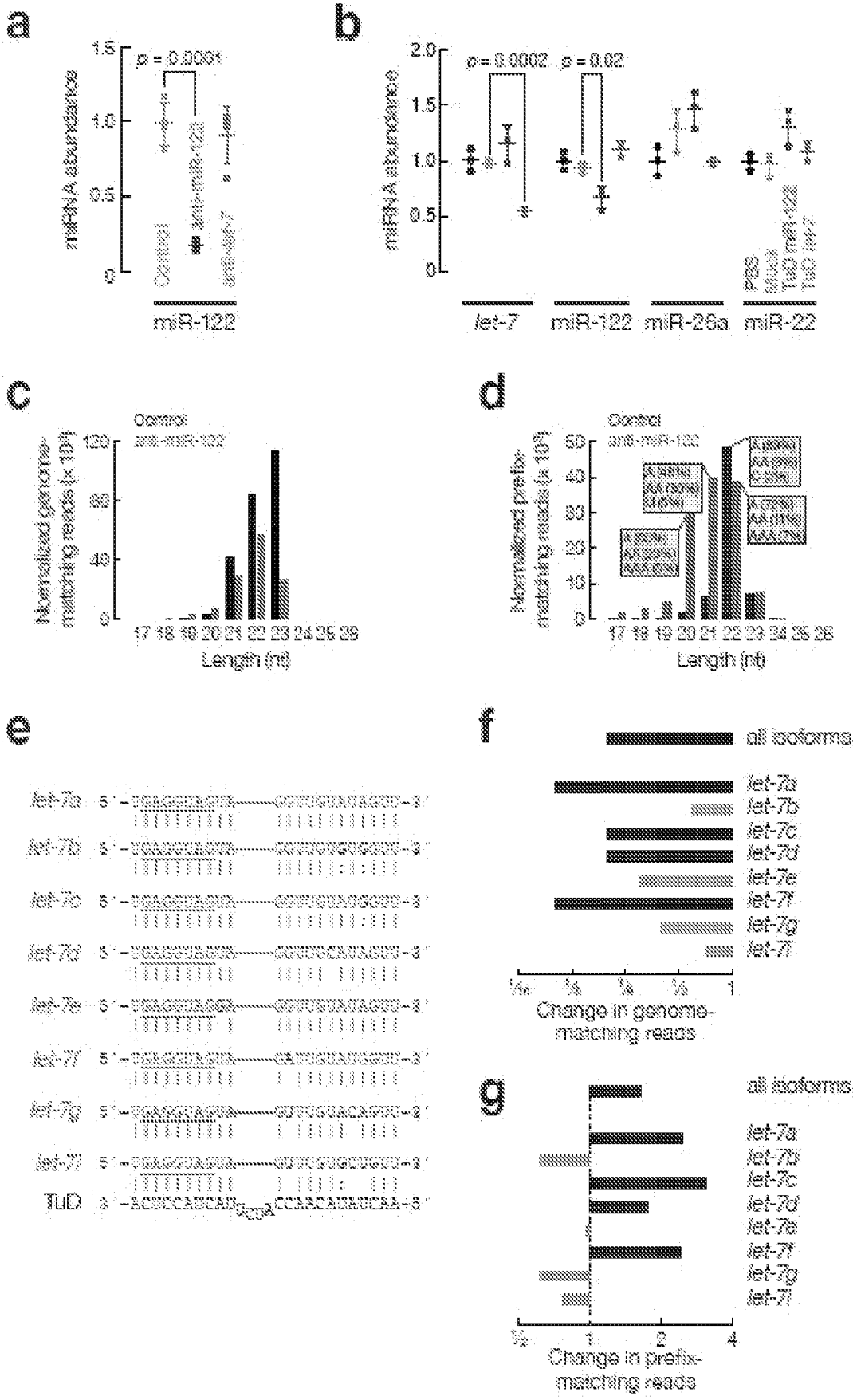

a
b
c
d
e
f
g
miRNA abundance
p = 0.0001
Control
anti-miR-122
anti-let-7
miR-122
p = 0.0002
p = 0.02
let-7
miR-26a
miR-22
Normalized genome-matching reads (x 10^6)
Length (nt)
Normalized prefix-matching reads (x 10^6)
let-7a
let-7b
let-7c
let-7d
let-7e
let-7f
let-7g
let-7i
TuD
all isoforms
Change in genome-matching reads
Change in prefix-matching reads

FIGURE 11

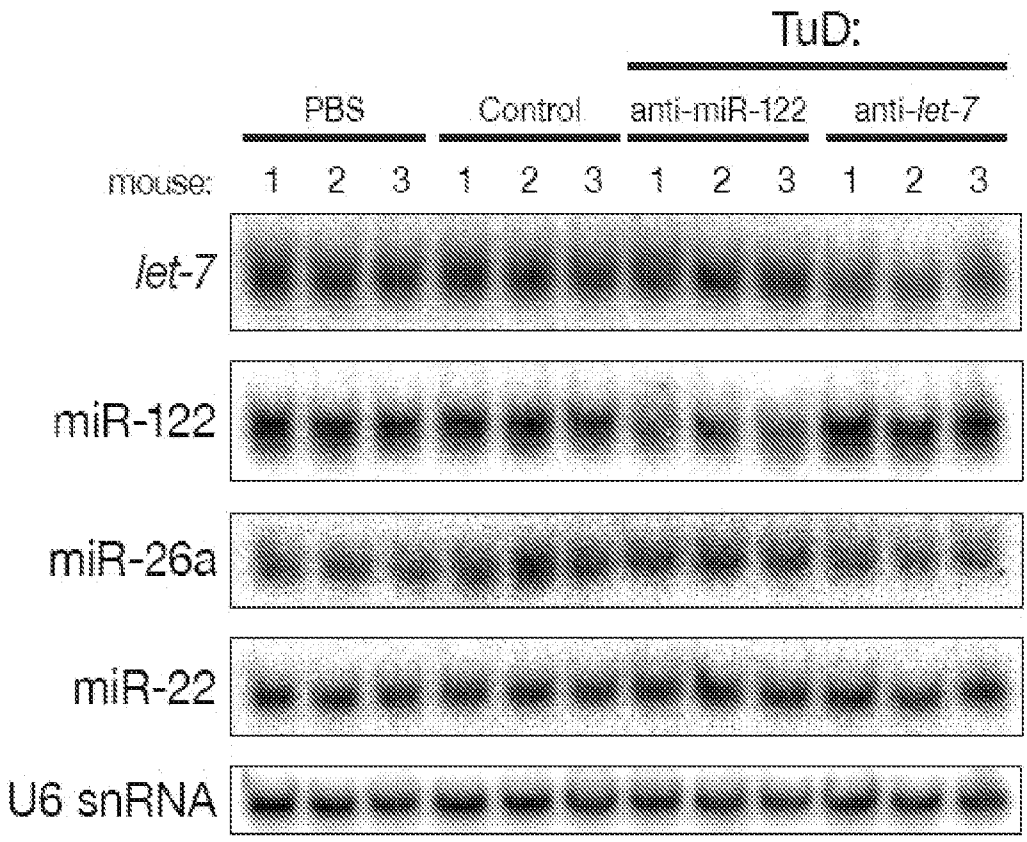

TuD:
PBS
Control
anti-miR-122
anti-let-7
mouse: 1 2 3 1 2 3 1 2 3 1 2 3
let-7
miR-122
miR-26a
miR-22
U6 snRNA

FIGURE 12

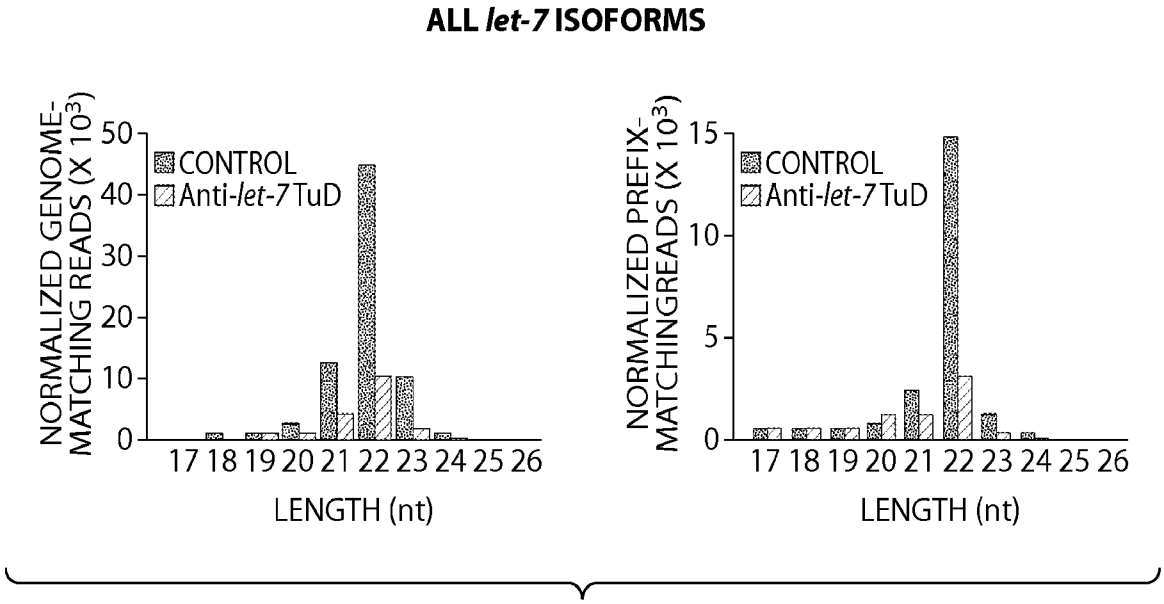

ALL let-7 ISOFORMS
NORMALIZED GENOME-MATCHING READS (X 10³)
50
40
30
20
10
0
CONTROL
Anti-let-7 TuD
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHINGREADS (X 10³)
15
10
5
0
CONTROL
Anti-let-7 TuD
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)

Fig. 13-1

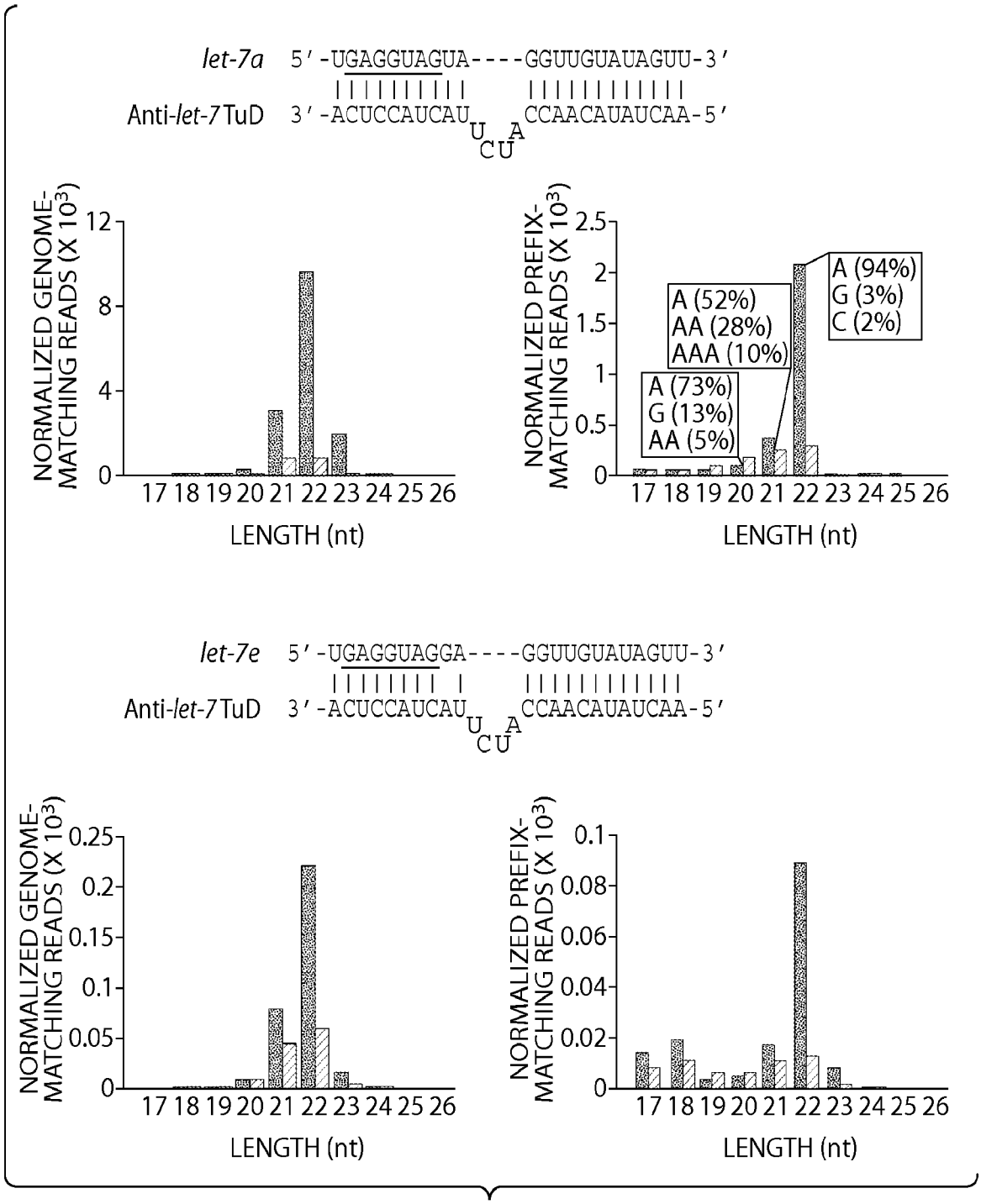

let-7a 5′-UGAGGUAGUA----GGUUGUAUAGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
CU
NORMALIZED GENOME-MATCHING READS (X 10³)
12
8
4
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
2.5
2
1.5
1
0.5
0
A (52%)
AA (28%)
AAA (10%)
A (73%)
G (13%)
AA (5%)
A (94%)
G (3%)
C (2%)
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
let-7e 5′-UGAGGUAGGA----GGUUGUAUAGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
CU
NORMALIZED GENOME-MATCHING READS (X 10³)
0.25
0.2
0.15
0.1
0.05
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
0.1
0.08
0.06
0.04
0.02
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)

Fig. 13-2

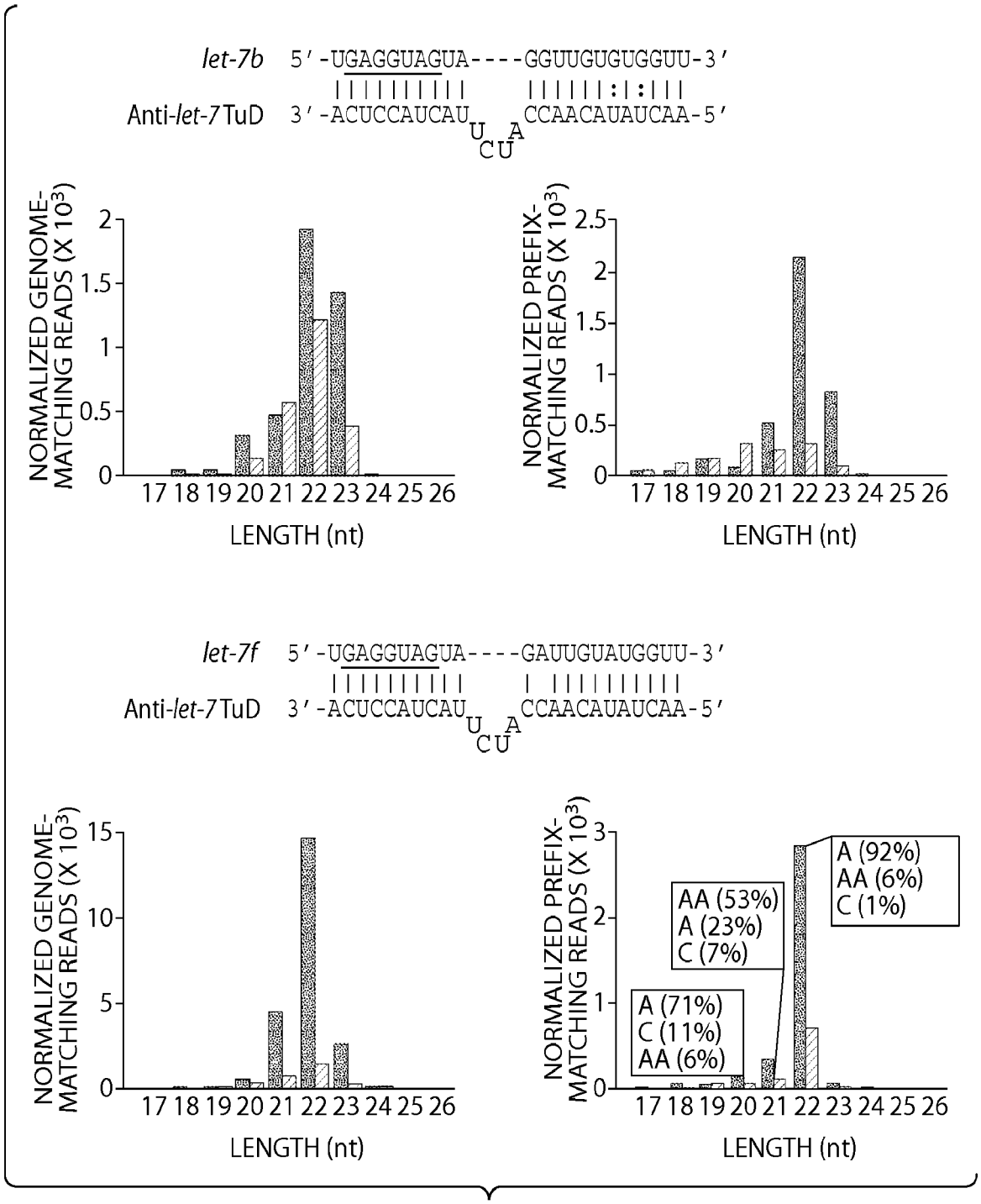

let-7b 5′-UGAGGUAGUA----GGUUGUGUGGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
NORMALIZED GENOME-MATCHING READS (X 10³)
NORMALIZED PREFIX-MATCHING READS (X 10³)
LENGTH (nt)
let-7f 5′-UGAGGUAGUA----GAUUGUAUGGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
A (92%)
AA (6%)
C (1%)
AA (53%)
A (23%)
C (7%)
A (71%)
C (11%)
AA (6%)

Fig. 13-3

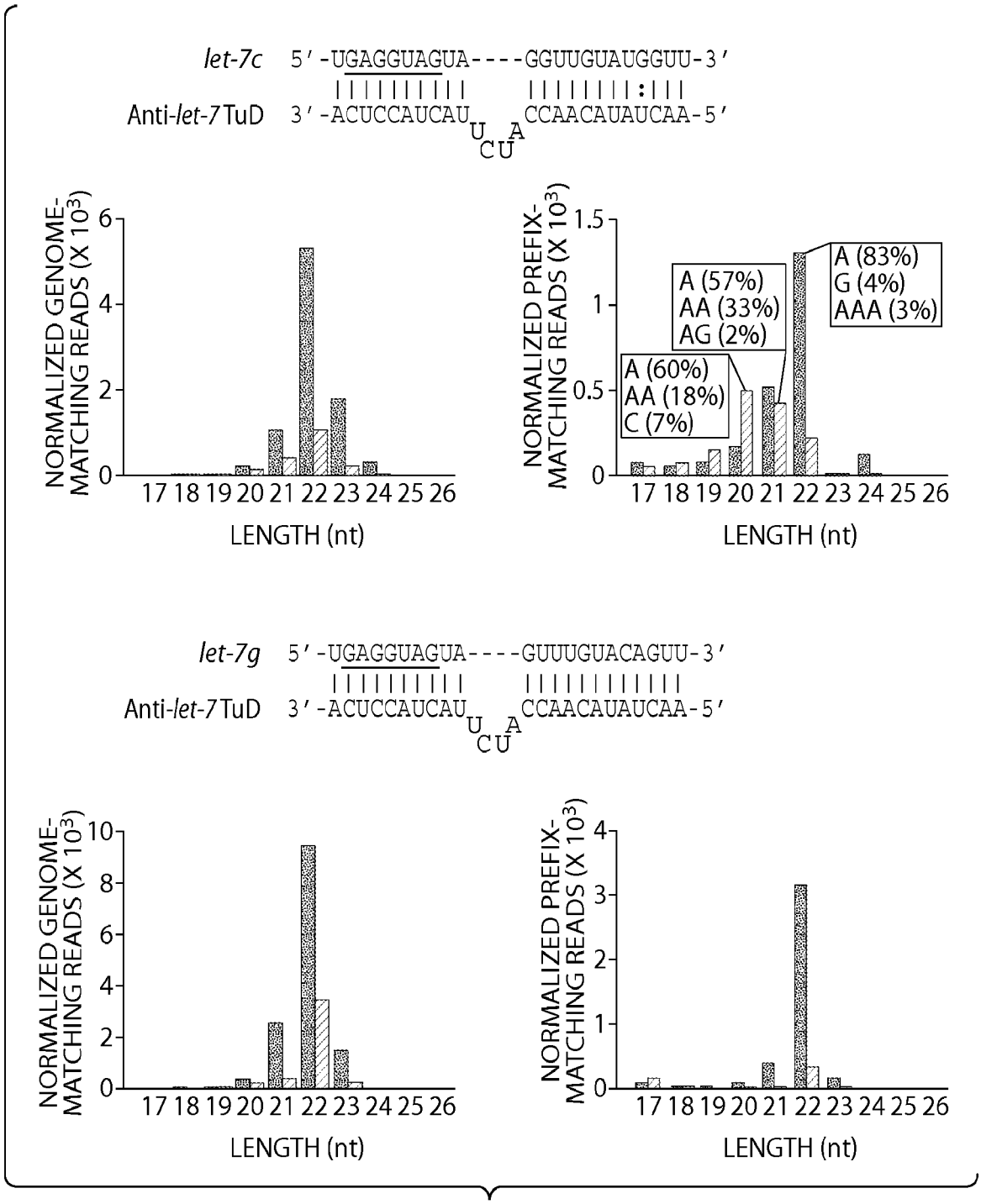

let-7c 5′-UGAGGUAGUA----GGUUGUAUGGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
NORMALIZED GENOME-MATCHING READS (X 10³)
0 2 4 6
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
0 0.5 1 1.5
A (57%)
AA (33%)
AG (2%)
A (83%)
G (4%)
AAA (3%)
A (60%)
AA (18%)
C (7%)
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
let-7g 5′-UGAGGUAGUA----GUUUGUACAGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
NORMALIZED GENOME-MATCHING READS (X 10³)
0 2 4 6 8 10
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
0 1 2 3 4
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)

Fig. 13-4

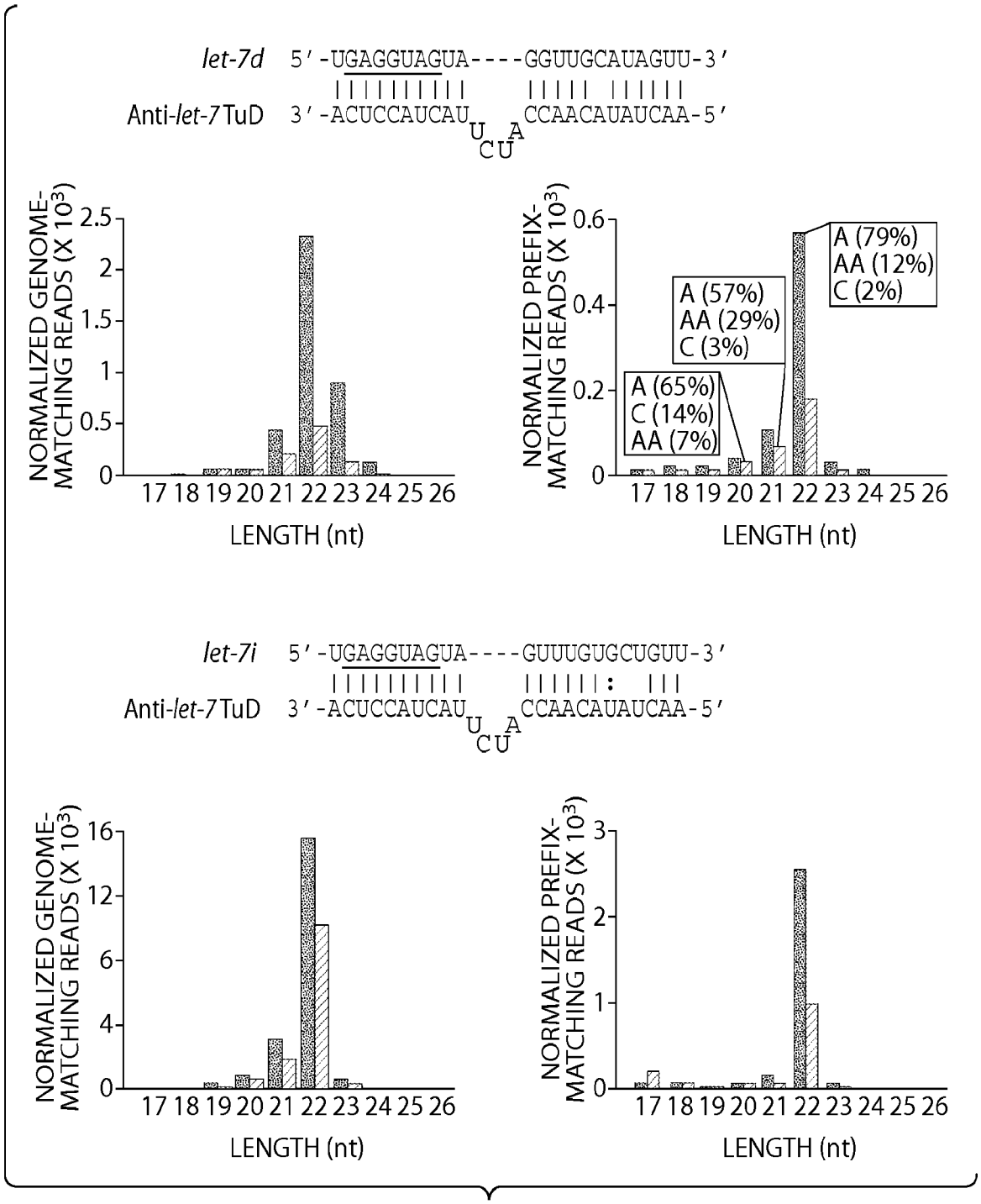

let-7d 5′-UGAGGUAGUA----GGUUGCAUAGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
NORMALIZED GENOME-MATCHING READS (X 10³)
2.5
2
1.5
1
0.5
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
0.6
0.4
0.2
0
A (79%)
AA (12%)
C (2%)
A (57%)
AA (29%)
C (3%)
A (65%)
C (14%)
AA (7%)
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
let-7i 5′-UGAGGUAGUA----GUUUGUGCUGUU-3′
Anti-let-7TuD 3′-ACUCCAUCAU U A CCAACAUAUCAA-5′
C U
NORMALIZED GENOME-MATCHING READS (X 10³)
16
12
6
4
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)
NORMALIZED PREFIX-MATCHING READS (X 10³)
3
2
1
0
17 18 19 20 21 22 23 24 25 26
LENGTH (nt)

Fig. 13-5

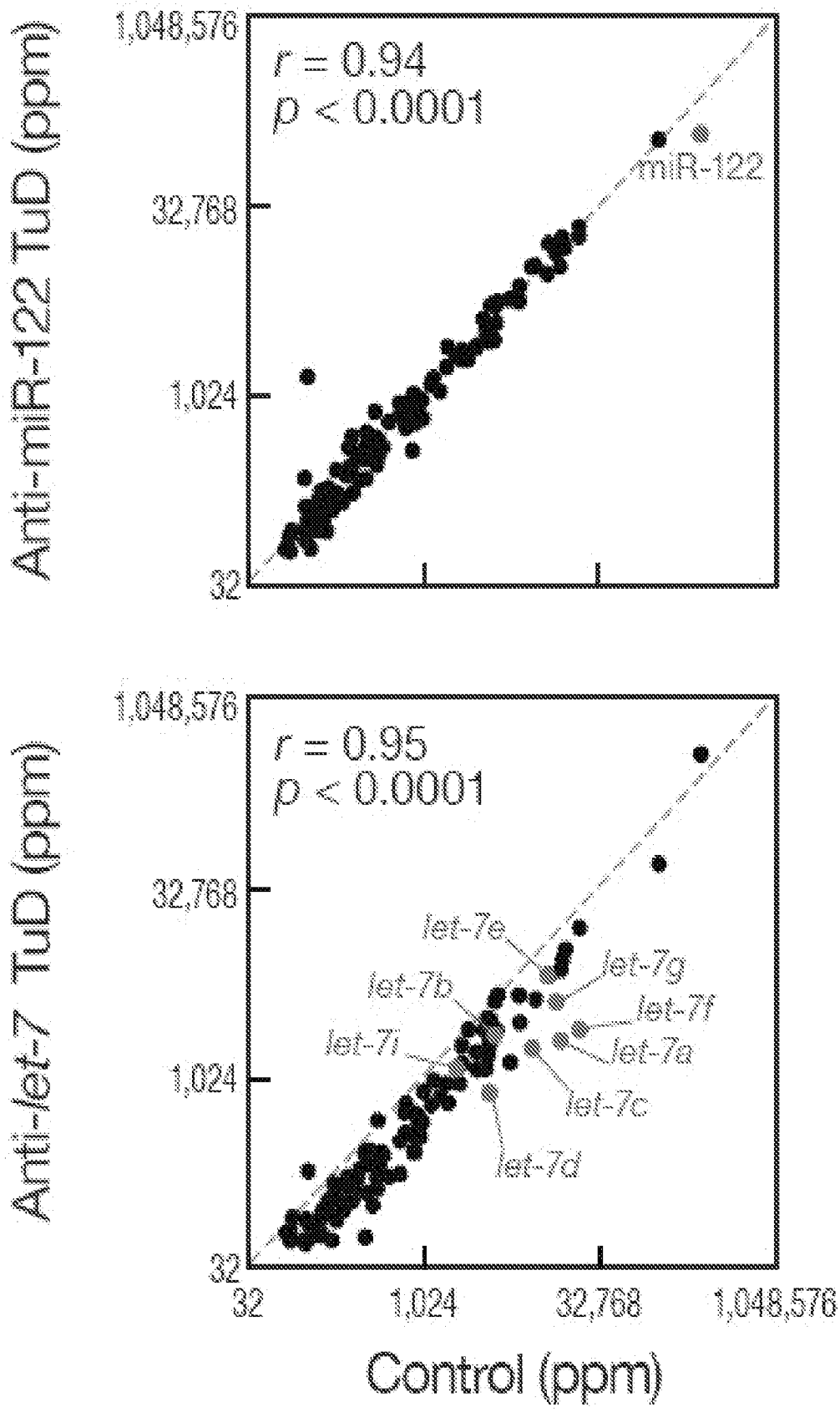

Anti-miR-122 TuD (ppm)
1,048,576
32,768
1,024
32
r = 0.94
p < 0.0001
miR-122
Anti-let-7 TuD (ppm)
1,048,576
32,768
1,024
32
r = 0.95
p < 0.0001
let-7e
let-7g
let-7b
let-7f
let-7i
let-7a
let-7c
let-7d
32
1,024
32,768
1,048,576
Control (ppm)

FIGURE 14

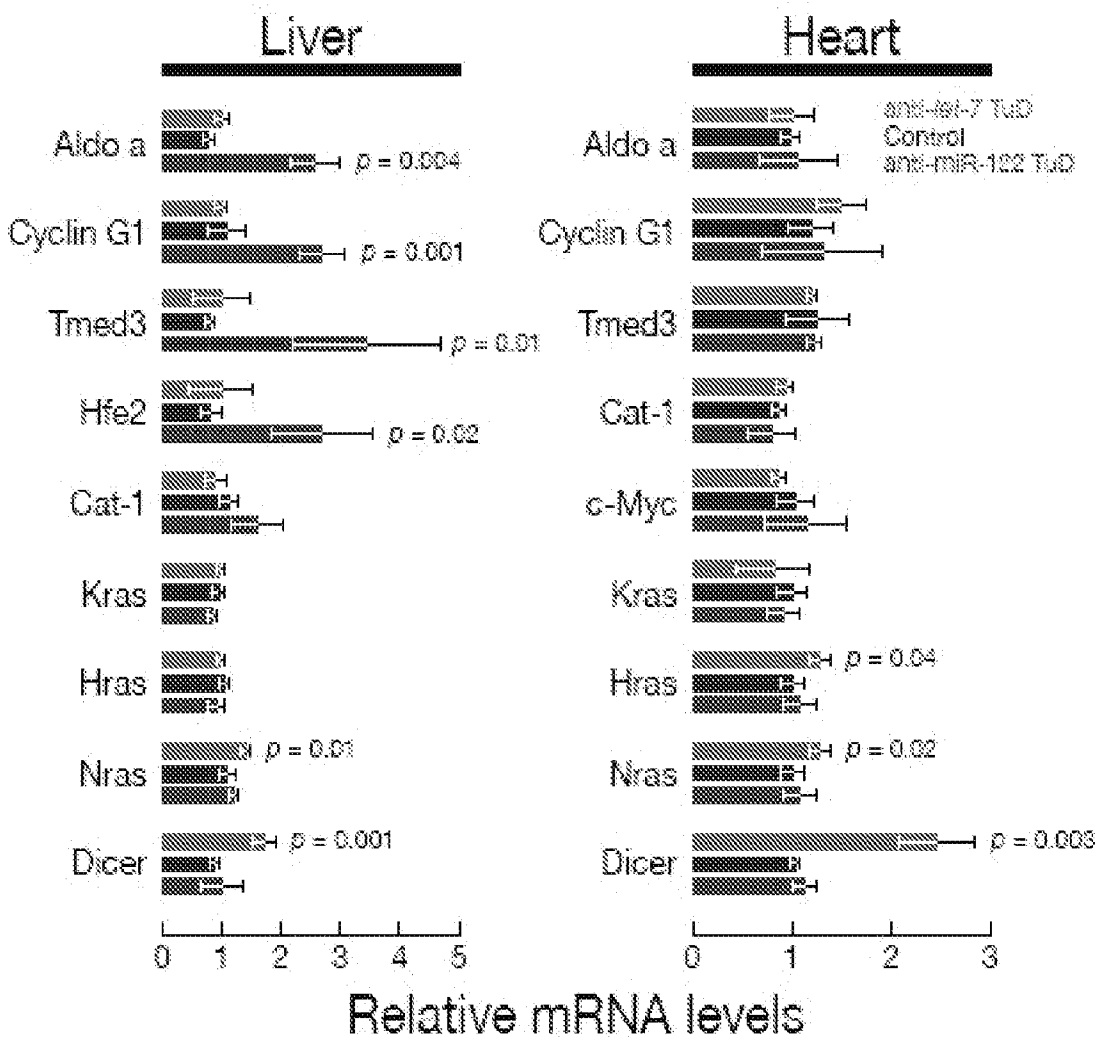

Liver
Heart
Aldo a
Cyclin G1
Tmed3
Hfe2
Cat-1
Kras
Hras
Nras
Dicer
c-Myc
p = 0.004
p = 0.001
p = 0.01
p = 0.02
p = 0.01
p = 0.001
p = 0.04
p = 0.02
p = 0.003
anti-let-7 TuD
Control
anti-miR-122 TuD
0 1 2 3 4 5
0 1 2 3
Relative mRNA levels

FIGURE 15

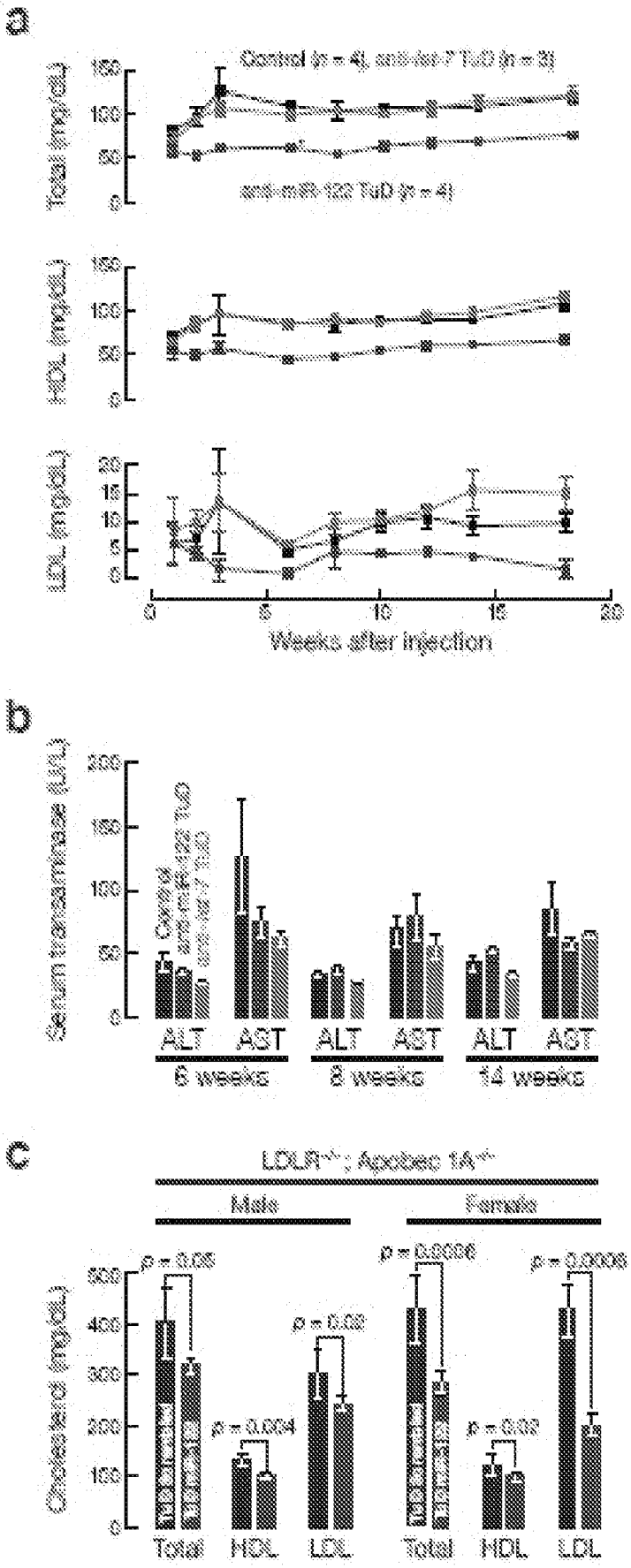

a
Total (mg/dL)
HDL (mg/dL)
LDL (mg/dL)
Weeks after injection
b
Serum transaminase (U/L)
ALT
AST
6 weeks
8 weeks
14 weeks
c
LDLR-/-; ApoBec 1A-/-
Male
Female
Cholesterol (mg/dL)
p = 0.05
p = 0.004
p = 0.02
p = 0.0006
p = 0.02
p = 0.0008
Total
HDL
LDL

FIGURE 16

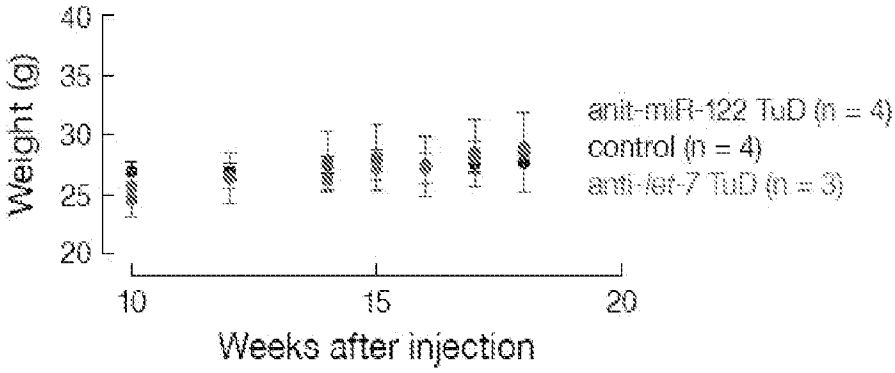

Weight (g)
40
35
30
25
20
10
15
20
Weeks after injection
anit-miR-122 TuD (n = 4)
control (n = 4)
anti-let-7 TuD (n = 3)

FIGURE 17