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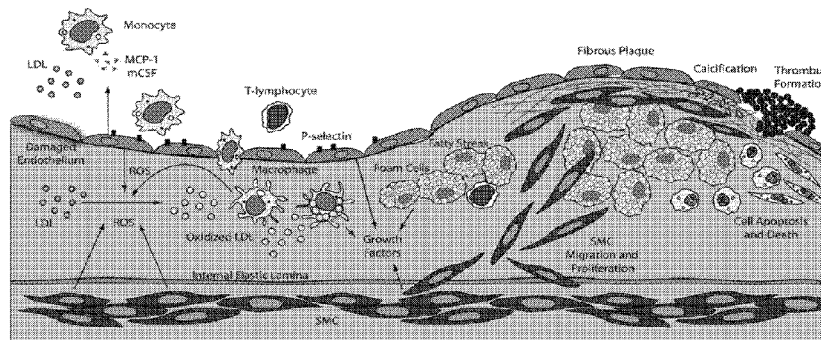
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(54) **Title:** METHOD TO MODULATE SMOOTH MUSCLE CELL DIFFERENTIATION

Figure 1

Development of Atherosclerosis

(57) **Abstract:** Disclosed herein are a methods of treating vascular disease or tumor growth, or inhibiting neovascularization in a mammal, comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the mammal, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor. Also disclosed herein are methods of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-25, comprising contacting the cell in vivo or in vitro with a miR-25 inhibitor. Also disclosed herein are medical devices comprising a drug coating comprising a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.

METHOD TO MODULATE SMOOTH MUSCLE CELL DIFFERENTIATION**CROSS-REFERENCE TO RELATED APPLICATIONS**

This Application claims the benefit of U.S. Provisional Application No. 62/257,594, filed November 19, 2015, which is incorporated herein by reference in its
5 entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under 101 BX001729 awarded by the Department of Veterans Affairs. The government has certain rights in the invention.

INCORPORATION OF THE SEQUENCE LISTING

The Sequence Listing submitted November 18, 2016 as a text filed named "37759_0001P1_Sequence_Listing.txt," created on November 17, 2016, and having a size of 4,096 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND OF THE INVENTION

15 Vascular smooth muscle cells (VSMC) are found within, and composing the majority of the wall of, blood vessels. Vascular smooth muscle contracts or relaxes to both change the volume of blood vessels and local blood pressure. This activity redistributes the blood within the body to areas where it is needed, and regulates the caliber of the blood vessels in the body. VSMCs show different phenotypes according to external conditions (e.g.,
20 developmental stage, angiogenesis state, and disease). Synthetic (dedifferentiated) smooth muscle cells have less contractive ability than contractile (differentiated) smooth muscle cells because of the lack of sufficient myofibrils inside the cells. Synthetic smooth muscle cells have the ability to proliferate and migrate, and they actively synthesize proteins and secrete extracellular matrices such as collagen and elastin.

25 MicroRNAs are involved in a number of biological processes including regulation of developmental timing, apoptosis, fat metabolism, and hematopoietic cell differentiation among others. MicroRNAs (miRs) are small, non-protein coding RNAs of about 18 to about 25 nucleotides in length that are derived from individual miRNA genes, from introns of protein coding genes, or from poly-cistronic transcripts that often encode multiple, closely
30 related miRNAs. MiRs act as repressors of target mRNAs by promoting their degradation, when their sequences are perfectly complementary, or by inhibiting translation, when their sequences contain mismatches. miRNAs are transcribed by RNA polymerase II (pol II) or

RNA polymerase III (pol III) and arise from initial transcripts, termed primary miRNA transcripts (pri-miRNAs), which are generally several thousand bases long. Pri-miRNAs are processed in the nucleus by the RNase Drosha into about 70- to about 100-nucleotide hairpin-shaped precursors (pre-miRNAs). Following transport to the cytoplasm, the hairpin pre-miRNA is further processed by Dicer to produce a double-stranded miRNA (also referred to as an siRNA). The mature single stranded miRNA guide strand is then incorporated into the RNA-induced silencing complex (RISC), where it associates with its target mRNAs by base-pair complementarity. In the relatively rare cases in which a miRNA base pairs perfectly with an mRNA target, it promotes mRNA degradation. More commonly, miRNAs form imperfect heteroduplexes with target mRNAs, affecting either mRNA stability or inhibiting mRNA translation.

The 5' portion of a miRNA spanning bases 2-8, termed the "seed" region, is especially important for target recognition. The sequence of the seed, together with phylogenetic conservation of the target sequence, forms the basis for many current target prediction models. Although increasingly sophisticated computational approaches to predict miRNAs and their targets are becoming available, target prediction remains a major challenge and requires experimental validation. Ascribing the functions of miRNAs to the regulation of specific mRNA targets is further complicated by the ability of individual miRNAs to base pair with hundreds of potential high and low affinity mRNA targets and by the targeting of multiple miRNAs to individual mRNAs.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention provides a method of treating vascular disease or tumor growth, or inhibiting neovascularization in a mammal, comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the mammal, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.

In certain embodiments, the therapeutic agent increases or maintains Nox4 NADPH oxidase expression in a cell in the mammal, as compared to Nox4 NADPH oxidase expression in a cell in a mammal that was not administered the therapeutic agent.

In certain embodiments, the levels of ROS are increases or unchanged as compared to ROS levels in a cell in a mammal that was not administered the therapeutic agent.

In certain embodiments, the myocardin mRNA expression is increased or unchanged as compared to myocardin mRNA expression in a cell in a mammal that was not administered the therapeutic agent.

5 In certain embodiments, the cell migration is reduced or prevented as compared to cell migration in a cell in a mammal that was not administered the therapeutic agent.

In certain embodiments, the vascular disease is atherosclerosis, bypass graft stenosis, angioplasty/stent restenosis.

In certain embodiments, the tumor is a gastrointestinal, bladder, or uterine tumor.

10 In certain embodiments, the inhibitor reduces miR-9 or miR-25 expression and/or function.

In certain embodiments, the miR-9 or miR-25 expression and/or function is reduced by at least 10%.

In certain embodiments, the inhibitor is a miR-9 or miR-25 inhibitor.

15 In certain embodiments, the miR-9 inhibitor is a miR-9 inhibitor comprising an oligonucleotide comprising 4 to 7 nucleotides that are complementary to miR-9 seed sequence 3'-UGGUUUC-5' (SEQ ID NO: 9).

In certain embodiments, the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to miR-9 seed sequence 3'-UGGUUUC-5' (SEQ ID NO: 9).

20 In certain embodiments, the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity with miR-9 3'-UCUUUGGUUAUCUAGCUGUAUGA-5' (SEQ ID NO: 2).

25 In certain embodiments, the miR-25 inhibitor is a miR-25 inhibitor comprising an oligonucleotide comprising 4 to 7 nucleotides that are complementary to miR-25 seed sequence 3'-CACGUUA-5' (SEQ ID NO: 7).

In certain embodiments, the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to miR-25 seed sequence 3'-CACGUUA-5' (SEQ ID NO: 7).

30 In certain embodiments, the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity with miR-25 3'-CAUUGCACUUGUCUCGGUCUGA-5' (SEQ ID NO: 8).

In certain embodiments, the oligonucleotide is between about 18 to about 25 nucleotides in length.

In certain embodiments, the oligonucleotide is chemically modified.

In certain embodiments, the oligonucleotide contains at least one modified nucleotide analog.

In certain embodiments, the modified nucleotide analog is a 2' modified nucleotide.

5 In certain embodiments, the modified nucleotide analog is a backbone-modified nucleotide.

In certain embodiments, the oligonucleotide contains at least one locked nucleic acid.

In certain embodiments, the administration is by systemic or local release.

In certain embodiments, the delivery is by intravenous delivery.

10 In certain embodiments, the present invention provides a method of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-9, comprising contacting the cell in vivo or in vitro with a miR-9 inhibitor.

In certain embodiments, the present invention provides a method of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-25, comprising contacting
15 the cell in vivo or in vitro with a miR-25 inhibitor.

In certain embodiments, the present invention provides a medical device comprising a drug coating comprising a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.

20 Disclosed herein are methods of treating vascular disease in a subject. The method can comprise administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

25 Disclosed herein are methods of reducing or inhibiting tumor growth in a subject. The method can comprise administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

30 Disclosed herein are methods of inhibiting neovascularization in a subject. The method can comprise administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

Disclosed herein are methods of preventing or reducing the risk of graft disease in a subject. The method can comprise administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

5 Disclosed herein are methods of preventing or reducing the risk of restenosis in a subject. The method can comprise administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

10 Disclosed herein are methods of preventing or reducing the risk of arterial restenosis or accelerated venous bypass disease in a subject. The method can comprise administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

Disclosed herein are articles of manufacture, comprising a solid substrate coated with a therapeutic agent comprising a micro-RNA-9 (miR-9) inhibitor and a micro-RNA-25 (miR-25) inhibitor. In some aspects, the therapeutic agent is a micro-RNA-9 (miR-9) inhibitor or a
15 micro-RNA-25 (miR-25) inhibitor. In some aspects, both a micro-RNA-9 (miR-9) inhibitor and a micro-RNA-25 (miR-25) inhibitor are coated on the solid support.

BRIEF DESCRIPTION OF THE FIGURES

20 FIG. 1 shows that smooth muscle cells de-differentiate in the vascular response to injury.

FIG. 2 shows myocardin-dependent vascular smooth muscle cell differentiation genes.

FIG.3 shows that Nox4 is required for maintenance of the differentiated vascular
25 smooth muscle cell phenotype.

FIG. 4 shows that microRNAs mediate changes in Nox4 expression and regulate expression of genes implicated in SMC differentiation.

FIG. 5 shows that miR-25 binds to Nox4 3'-UTR and silences gene expression.

FIG. 6 shows that miR-25 silences Nox4 expression.

30 FIG. 7 shows that Nox4 and miR-25 regulate myocardin expression.

FIG. 8 shows that miR-25 induces miR-9 expression in SMCs.

FIG.9 shows that miR-9 binds to the myocardin 3'-UTR. COS7 cells were transfected with WT or miR-9 mutant myocardin 3'-UTR luciferase, then with the miR-9 or miR-25 mimics. Renilla RLU is normalized to Firefly RLU, $p<0.05$ vs WT, $n=3$.

FIG. 10 shows that miR-9 binds to Nox4 3'-UTR at two sites.

FIG. 11 shows that miR-25 causes demethylation of the miR-9 promoter. Human SMCs were transfected with miR-25, then genomic DNA was bisulfite converted and amplified. Sequences were obtained by TA cloning of the PCR transcripts into PGEM T-Easy and sequenced using capillary sequencing. Ratios of methylated I total clones for each CpG site analyzed are shown above. Control Cones: 7, miR-25 Clones: 9. F-Value:6.08, $*p<0.05$.
1-4 miR-9-1, 5-8 miR-9-2, 9- 12 miR-9-3. 1,2,5,6,9,10 unmethylated. 3,4,7,8,11,12 methylated.

FIG.12 is a schematic showing a pathway by which miR-25 silences Nox4 and causes hypomethylation of the miR-9 promoter, increasing the expression of miR-9. MiRNA-9 silences Nox4 and myocardin and in doing so allows for the de-differentiation of vascular smooth muscle cells which is necessary for the development of vascular disease. Inhibiting the function of miR-25 and/or miR-9 can maintain SMC differentiation and attenuates vascular disease.

FIG. 13 shows the effect of growth factors and cytokines on miR-9 and miR-25 levels.

FIG. 14 shows that thrombin-mediated increase in miR-9 silences myocardin expression in HEK293 cells.

FIG. 15 shows that miR-9 and miR-25 cause de-differentiation of SMCs.

FIG. 16 shows that miR-9 and miR-25 levels are increased in SMCs derived from the neointima.

FIG. 17 shows that miR-9 is increased in injured carotid artery and miR-9 inhibitor decreases intimal formation.

FIG. 18 shows that miR-9/Nox4 are part of a novel regulatory pathway.

FIG. 19 shows that relative expression of miR-9 (left) and miR-25 (right) are increased in injured carotid artery, ten days post ligation.

FIG. 20 shows that arteries treated with a miR-9 inhibitor decreases intimal formation.

DETAILED DESCRIPTION

The present disclosure can be understood more readily by reference to the following detailed description of the invention, the figures and the examples included herein.

Before the present compositions and methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular
5 aspects only and is not intended to be limiting. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, example methods and materials are now described.

Moreover, it is to be understood that unless otherwise expressly stated, it is in no way intended that any method set forth herein be construed as requiring that its steps be performed
10 in a specific order. Accordingly, where a method claim does not actually recite an order to be followed by its steps or it is not otherwise specifically stated in the claims or descriptions that the steps are to be limited to a specific order, it is in no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain
15 meaning derived from grammatical organization or punctuation, and the number or type of aspects described in the specification.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The publications discussed herein are provided solely for their disclosure prior to the filing
20 date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided herein can be different from the actual publication dates, which can require independent confirmation.

25 NOX4

NADPH Oxidase 4 is an enzyme encoded by the *NOX4* gene. The NOX4 enzyme is responsible for oxygen sensing, and protects the vasculature against inflammatory stress. Studies have shown that the pharmacological inhibition of NOX4 reverses or prevents
NOX4-associated vascular inflammation and remodeling.

30 miRs

The present invention is based on the discovery that miR-25 and miR-9 are involved in the regulation of smooth muscle cell dedifferentiation in vascular disease. Accordingly, the present invention provides a method of treating vascular disease in a subject in need

thereof comprising identifying a subject having vascular disease; and administering to the subject a functional miR-9 and/or miR-25 inhibitor. Disclosed here are methods of treating vascular disease in a subject in need thereof comprising identifying a subject having vascular disease; and administering to the subject a therapeutically effective (e.g., functional) miR-9 and/or miR-25 inhibitor. The miR inhibitor may be administered by parenteral administration (e.g., intravenous or subcutaneous), transdermal, sustained release, controlled release, delayed release, catheter or sublingual administration. In certain embodiments, the miR inhibitor is coated onto a solid substrate, such as an implanted device (e.g., a drug eluting arterial stent). By treatment is meant that at least an amelioration of the symptoms associated with the condition afflicting the host is achieved, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. symptom, associated with the condition being treated. As such, treatment also includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited, e.g. prevented from happening, or stopped, e.g. terminated, such that the host no longer suffers from the condition, or at least the symptoms that characterize the condition.

The present invention also contemplates a method of treating vascular disease in a subject comprising identifying a subject having vascular disease and decreasing the expression and/or activity of miR-9 and/or miR-25 in smooth muscle cells of the subject. In some embodiments, decreasing the expression and/or activity of miR-9 and/or miR-25 comprises administering a miR-9 and/or miR-25 inhibitor to the subject.

A miR-9 inhibitor may be a polynucleotide comprising the sequence of a mature miR-9 sequence. The miR-9 inhibitor may also be an expression vector encoding miR-9. The miR9 inhibitor may also be an expression vector encoding pre- miR-9. The miR-9 inhibitor may also be an expression vector encoding pre-miR-9 or pri-miR-9. In certain embodiments, the miR-9 inhibitor is an artificial miRNA cassette that delivers the mature miR-9 guide strand.

A miR-25 inhibitor may be a polynucleotide comprising the sequence of a mature miR-25 sequence. The miR-25 inhibitor may also be an expression vector encoding miR-25. The miR-25 inhibitor may also be an expression vector encoding pre-miR-25. The miR-25 inhibitor may also be an expression vector encoding pre-miR9 or pri-miR-25. In certain embodiments, the miR-25 inhibitor is an artificial miRNA cassette that delivers the mature miR-25 guide strand.

As used herein, the term “miRNA guide strand” is a single-stranded, non-coding RNA that is complementary to a target sequence. As used herein, the term “miRNA non-guide

strand” is a single-stranded sequence of RNA that is complementary to the “miRNA guide strand.” In certain embodiments, the miRNA strand is about 20-30 nucleotides in length.

The present invention further provides an expression cassette containing a promoter contiguously linked to the nucleic acid described herein. In certain embodiments, the promoter is a pol II or a pol III promoter, such as a U6 promoter (e.g., a mouse U6 promoter). In certain embodiments, the expression cassette further contains a marker gene. In certain embodiments, the promoter is a pol II promoter. In certain embodiments, the promoter is a tissue-specific promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiments, the promoter is a pol III promoter.

The present invention provides a vector containing an expression cassette described herein. In certain embodiments, the vector is an adeno-associated virus (AAV) vector.

The present invention also provides vectors containing the expression cassettes described herein. Examples of appropriate vectors include adenoviral, lentiviral, adeno-associated viral (AAV), poliovirus, herpes simplex virus (HSV), or murine Maloney-based viral vectors. In one embodiment, the vector is an adeno-associated virus vector. These cassettes and vectors may be contained in a cell, such as a mammalian cell. A non-human mammal may contain the cassette or vector.

The present invention provides cells (such as a mammalian cell) containing the nucleic acid molecules, expression cassettes or vectors described herein. The present invention also provides a non-human mammal containing the nucleic acid molecules, expression cassettes or vectors described herein.

The present invention provides a nucleic acid, an expression cassette, a vector, or a composition as described herein for use in therapy, such as for treating vascular disease.

The present invention provides a method of treating a subject with vascular disease by administering to the subject a nucleic acid, an expression cassette, a vector, or a composition as described herein so as to treat the vascular disease.

The present invention provides a viral vector comprising a promoter and a microRNA (miRNA) shuttle containing an embedded siRNA specific for a target sequence. In certain embodiments, the promoter is an inducible promoter. In certain embodiments, the vector is an adenoviral, lentiviral, adeno-associated viral (AAV), poliovirus, HSV, or murine Maloney-based viral vector. In certain embodiments, the targeted sequence is a sequence associated with vascular disease.

This invention relates to compounds, compositions, and methods useful for modulating vascular disease gene expression using miRNA molecules. In particular, the

instant invention features small nucleic acid molecules, miR-9 and/or miR-25 inhibitors, and methods used to treat vascular disease. A miR-9 or miR-25 molecule or inhibitor of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized.

5 As used herein when a claim indicates an RNA “corresponding to” it is meant the RNA that has the same sequence as the DNA, except that uracil is substituted for thymine.

Methods of delivery of viral vectors include, but are not limited to, intra-arterial, intra-muscular, intravenous, intranasal and oral routes. Generally, virions may be introduced into cells using either in vivo or in vitro transduction techniques. If transduced in vitro, the
10 desired recipient cell will be removed from the subject, transduced with virions and reintroduced into the subject. Alternatively, syngeneic or xenogeneic cells can be used where those cells will not generate an appropriate immune response in the subject.

Suitable methods for the delivery and introduction of transduced cells into a subject have been described. For example, cells can be transduced in vitro by combining
15 recombinant virions with target cells e.g., in appropriate media, and screening for those cells harboring the DNA of interest can be screened using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, described more fully below, and the composition introduced into the subject by various techniques, such as by grafting,
20 intramuscular, intravenous, subcutaneous and intraperitoneal injection.

In one embodiment, for in vivo delivery, virions are formulated into pharmaceutical compositions and will generally be administered parenterally, e.g., by injection directly into smooth muscle.

In one embodiment, viral vectors of the invention are delivered to the liver via
25 convection-enhanced delivery (CED) systems that can efficiently deliver viral vectors, e.g., AAV. As described in detail and exemplified below, these methods are suitable for a variety of viral vectors, for instance AAV vectors carrying therapeutic genes (e.g., miR-9 or miR-25 inhibitors).

Any convection-enhanced delivery device may be appropriate for delivery of viral
30 vectors. In one embodiment, the device is an osmotic pump or an infusion pump. Both osmotic and infusion pumps are commercially available from a variety of suppliers, for example Alzet Corporation, Hamilton Corporation, and Aiza, Inc.. Typically, a viral vector is delivered via CED devices as follows. A catheter, cannula or other injection device is inserted into liver tissue in the chosen subject. Stereotactic maps and positioning devices are

available, for example from ASI Instruments, Warren, Mich. Positioning may also be conducted by using anatomical maps obtained by CT and/or MRI imaging of the subject's liver to help guide the injection device to the chosen target. Moreover, because the methods described herein can be practiced such that relatively large areas of the liver take up the viral
5 vectors, fewer infusion cannula are needed. Since surgical complications are related to the number of penetrations, the methods described herein also serve to reduce the side effects seen with conventional delivery techniques.

In one embodiment, pharmaceutical compositions can comprise sufficient genetic material to produce a therapeutically effective amount of miR-9 or miR-25 inhibitors, i.e., an
10 amount sufficient to reduce or ameliorate symptoms of vascular disease or an amount sufficient to confer the desired benefit. The pharmaceutical compositions may also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically
15 acceptable excipients include, but are not limited to, sorbitol, Tween80, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying
20 agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991).

As is apparent to those skilled in the art in view of the teachings of this specification, an effective amount of viral vector which must be added can be empirically determined.

25 Administration can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosages of administration are well known to those of skill in the art and will vary with the viral vector, the composition of the therapy, the target cells, and the subject being treated. Single and multiple administrations can be carried out with the dose level and pattern being selected by
30 the treating physician.

It should be understood that more than one miR-9 or miR-25 inhibitor sequence could be expressed by the delivered viral vector. The miR-9 or miR-25 inhibitor could be expressed from a single vector. Alternatively, separate vectors, each expressing one or more different miR inhibitor sequences, can also be delivered to the liver as described herein.

Furthermore, it is also intended that the viral vectors delivered by the methods of the present invention be combined with other suitable compositions and therapies.

The present invention further provides a miR-9 and/or miR-25 inhibitor, an expression cassette and/or a vector as described herein for use in medical treatment.

5 The present invention provides the use of a miR-9 and/or miR-25 inhibitor, an expression cassette and/or a vector as described herein to prepare a medicament useful for treating vascular disease.

The present invention also provides a nucleic acid, expression cassette, vector, or composition of the invention for use in therapy.

10 The present invention also provides a nucleic acid, expression cassette, vector, or composition of the invention for treating, e.g., for use in the therapeutic treatment of vascular disease.

The present invention provides methods of reducing the amount of miR-9 and/or miR-25 in a target cell, where the target cell may be present in vitro or in vivo. By "reducing the amount of" is meant that the level or quantity of the target miR-9 and/or miR-25 in the target cell is reduced by at least about 2-fold, usually by at least about 5-fold, e.g., 10-fold, 15-fold, 20-fold, 50-fold, 100-fold or more, as compared to a control, i.e., an identical target cell not treated according to the subject methods.

15 In practicing the subject methods, an effective amount of miR-9 and/or miR-25 inhibitor is introduced into the target cell, where any convenient protocol for introducing the agent into the target cell may be employed. As is known in the art, miRNAs are single stranded RNA molecules that range in length from about 20 to about 25 nt, such as from about 21 to about 24 nt, e.g., 22 or 23 nt. The miRNA inhibitor may or may not be completely complementary to a region of the same length as the miRNA in the target viral genome. If not completely complementary, the miRNA and its corresponding target viral genome are at least substantially complementary, such that the amount of mismatches present over the length of the miRNA, (ranging from about 20 to about 25 nt) will not exceed about 8 nt, and will in certain embodiments not exceed about 6 or 5 nt, e.g., 4 nt.

30 **miR-9 and miR-25 Polynucleotide Inhibitors**

miR-9 miRNA is a member of the miRNA precursor family based on their sequence homology. The miR-9 microRNA (homologous to miR-79), is a short non-coding RNA gene involved in gene regulation. The mature ~21nt miRNAs are processed from hairpin precursor sequences by the Dicer enzyme. The dominant mature miRNA sequence is processed from

the 5' arm of the mir-9 precursor, and from the 3' arm of the mir-79 precursor. miR-9 expression levels implicated in human cancer, in particular breast cancer and clear cell renal carcinoma tumors. The mir-9 miRNA gene family includes 222 sequences. Since there are only minor differences between the family members, and the members have a 100% conserved seed region (which helps to define target determination), they are very likely to target the same mRNA targets, and lower gene expression of these specific target genes.

There are three known human mir-9 sequences:

> Stem-loop sequence hsa-mir-9-1 (MI0000466)

CGGGGUUGGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGGUGUGGAGUCUUCAUAAAGCU
10 AGAUAACCGAAAGUAAAAUAACCCCA (SEQ ID NO: 1)

> Mature sequence hsa-miR-9-5p (MIMAT0000441)

UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO: 2)

> Stem-loop sequence hsa-mir-9-2 (MI0000467)

GGAAGCGAGUUGUUAUCUUUGGUUAUCUAGCUGUAUGAGUGUAUUGGUCUUCAUAAAGCUAG
15 AUAACCGAAAGUAAAAACUCCUUCA (SEQ ID NO: 3)

> Mature sequence hsa-miR-9-5p

UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO: 2)

> Stem-loop sequence hsa-mir-9-3 (MI0000468)

GGAGGCCCGUUUCUCUCUUUGGUUAUCUAGCUGUAUGAGUGCCACAGAGCCGUCAUAAAGCU
20 AGAUAACCGAAAGUAGAAAUGAUUCUCA (SEQ ID NO: 4)

> Mature sequence hsa-miR-9-5p

UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO: 2)

25 miR-25 miRNA is another member of the miRNA precursor family. miR-25 levels increase in human heart failure and treatment with an anti-sense RNA molecule (antagomiR) has been shown to halt disease progression and improve cardiac function. There is one known human mir-25 sequence:

> Stem-loop sequence hsa-mir-25 (MI0000082)

GGCCAGUGUUGAGAGGCGGAGACUUGGGCAAUUGCUGGACGCUGCCCUGGGCAAUUGCACUUG
30 UCUCGGUCUGACAGUGCCGGCC (SEQ ID NO: 5)

> Mature sequence hsa-miR-25-5p (MIMAT0004498)

AGGCGGAGACUUGGGCAAUUG (SEQ ID NO: 6)

The present invention provides a method of treating vascular disease in a subject in need thereof comprising identifying a subject having vascular disease and administering to the subject a miR-9 and/or miR-25 inhibitor. In one embodiment, miR inhibitors may be polynucleotides comprising the mature miR sequence. In another embodiment, the miR inhibitor may be a polynucleotide comprising the pri-miRNA or pre-miRNA sequence for the miR. The polynucleotide comprising the mature miR-9, pre-miR-9, pri-miR-9, mature miR-25, pre-miR-25, pri-miR-25 sequence may be single stranded or double stranded. The polynucleotides may contain one or more chemical modifications, such as locked nucleic acids, peptide nucleic acids, sugar modifications, such as 2'-O-alkyl (e.g. 2'-O-methyl, 2'-O-methoxyethyl), 2'-fluoro, and 4' thio modifications, and backbone modifications, such as one or more phosphorothioate, morpholino, or phosphonocarboxylate linkages.

In one embodiment, the miR-9 or miR-25 inhibitor may be expressed in vivo from a vector. A "vector" is a composition of matter which can be used to deliver a nucleic acid of interest to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like. An expression construct can be replicated in a living cell, or it can be made synthetically. For purposes of this application, the terms "expression construct," "expression vector," and "vector," are used interchangeably to demonstrate the application of the invention in a general, illustrative sense, and are not intended to limit the invention.

In one embodiment, an expression vector for expressing the miR-9 or miR-25 inhibitor comprises a promoter "operably linked" to a polynucleotide encoding the miR-9 or miR-25 inhibitor, or combinations thereof. The phrase "operably linked" or "under transcriptional control" as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide. In another embodiment, the expression vector comprises a polynucleotide operably linked to a promoter, wherein the polynucleotide comprises the sequence encoding the miR inhibitor. In another embodiment, the expression vector comprises a polynucleotide operably linked to a promoter, wherein the polynucleotide comprises the miR inhibitor. The polynucleotide comprising the sequence of the miR inhibitor may be about 18 to about 2000 nucleotides in length, about 70 to about 200

nucleotides in length, about 20 to about 50 nucleotides in length, or about 18 to about 25 nucleotides in length.

In an aspect, the miR-9 and miR-25 inhibitors can be nucleic acid based. Nucleic acid therapeutic agents can be based on nucleic acids or closely related chemical compounds.

5 Examples of nucleic acid based therapeutics include but are not limited to antisense oligonucleotides, aptamers, small interfering RNAs, exon skipping, RNA editing, microRNA therapeutic inhibitors and mimics, long non-coding RNA modulators and mRNA.

In an aspect, the miR-9 and miR-25 inhibitors can be functional nucleic acids. In an aspect, the nucleic acid based therapeutic agent or functional nucleic acid therapeutic agent can be an
10 antisense molecule, aptamer, ribozyme, triplex forming molecules or an external guide sequence.

Functional nucleic acids are nucleic acid molecules that have a specific function, such as binding a target molecule or catalyzing a specific reaction. Functional nucleic acid molecules can be divided into the following categories, which are not meant to be limiting.

15 For example, functional nucleic acids include antisense molecules, aptamers, ribozymes, triplex forming molecules, and external guide sequences. The functional nucleic acid molecules can act as effectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional nucleic acid molecules can possess a *de novo* activity independent of any other molecules.

20 Functional nucleic acid molecules can interact with any macromolecule, such as DNA, RNA, polypeptides, or carbohydrate chains. Thus, functional nucleic acids can interact with the mRNA of polynucleotide sequences disclosed herein or the genomic DNA of the polynucleotide sequences disclosed herein or they can interact with the polypeptide encoded by the polynucleotide sequences disclosed herein. Often functional nucleic acids are
25 designed to interact with other nucleic acids based on sequence homology between the target molecule and the functional nucleic acid molecule. In other situations, the specific recognition between the functional nucleic acid molecule and the target molecule is not based on sequence homology between the functional nucleic acid molecule and the target molecule, but rather is based on the formation of tertiary structure that allows specific recognition to
30 take place.

Disclosed herein are antisense molecules that interact with the disclosed polynucleotides. Antisense molecules are designed to interact with a target nucleic acid molecule through either canonical or non-canonical base pairing. The interaction of the antisense molecule and the target molecule is designed to promote the destruction of the

target molecule through, for example, RNaseH mediated RNA-DNA hybrid degradation. Alternatively, the antisense molecule is designed to interrupt a processing function that normally would take place on the target molecule, such as transcription or replication. Antisense molecules can be designed based on the sequence of the target molecule.

- 5 Numerous methods for optimization of antisense efficiency by finding the most accessible regions of the target molecule exist. Exemplary methods would be *in vitro* selection experiments and DNA modification studies using DMS and DEPC. In an aspect, the antisense molecules bind the target molecule with a dissociation constant (k_d) less than or equal to 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . A representative sample of methods and techniques which
- 10 aid in the design and use of antisense molecules can be found in the following non-limiting list of United States patents: 5,135,917, 5,294,533, 5,627,158, 5,641,754, 5,691,317, 5,780,607, 5,786,138, 5,849,903, 5,856,103, 5,919,772, 5,955,590, 5,990,088, 5,994,320, 5,998,602, 6,005,095, 6,007,995, 6,013,522, 6,017,898, 6,018,042, 6,025,198, 6,033,910, 6,040,296, 6,046,004, 6,046,319, and 6,057,437 each of which is herein incorporated by
- 15 reference in its entirety for their teaching of modifications and methods related to the same.

Also disclosed are aptamers that interact with the disclosed polynucleotides.

- Aptamers are molecules that interact with a target molecule, preferably in a specific way. Typically aptamers are small nucleic acids ranging from 15-50 bases in length that fold into defined secondary and tertiary structures, such as stem-loops or G-quartets. Aptamers can
- 20 bind small molecules, such as ATP (United States patent 5,631,146) and theophiline (United States patent 5,580,737), as well as large molecules, such as reverse transcriptase (United States patent 5,786,462) and thrombin (United States patent 5,543,293). Aptamers can bind very tightly with k_d s from the target molecule of less than 10^{-12} M. In an aspect, the aptamers bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Aptamers can bind the
- 25 target molecule with a very high degree of specificity. For example, aptamers have been isolated that have greater than a 10000 fold difference in binding affinities between the target molecule and another molecule that differ at only a single position on the molecule (United States patent 5,543,293). In an aspect, the aptamer can have a k_d with the target molecule at least 10, 100, 1000, 10,000, or 100,000 fold lower than the k_d with a background binding
- 30 molecule. It is preferred when doing the comparison for a polypeptide for example, that the background molecule be a different polypeptide. For example, when determining the specificity of aptamers, the background protein could be ef-1 α . Representative examples of how to make and use aptamers to bind a variety of different target molecules can be found in

the following non-limiting list of United States patents: 5,476,766, 5,503,978, 5,631,146, 5,731,424, 5,780,228, 5,792,613, 5,795,721, 5,846,713, 5,858,660, 5,861,254, 5,864,026, 5,869,641, 5,958,691, 6,001,988, 6,011,020, 6,013,443, 6,020,130, 6,028,186, 6,030,776, and 6,051,698.

5 Also disclosed are ribozymes that interact with the disclosed polynucleotides. Ribozymes are nucleic acid molecules that are capable of catalyzing a chemical reaction, either intramolecularly or intermolecularly. Ribozymes are thus catalytic nucleic acid. Ribozymes catalyze intermolecular reactions. There are a number of different types of ribozymes that catalyze nuclease or nucleic acid polymerase type reactions which are based
10 on ribozymes found in natural systems, such as hammerhead ribozymes (for example, but not limited to the following United States patents: 5,334,711, 5,436,330, 5,616,466, 5,633,133, 5,646,020, 5,652,094, 5,712,384, 5,770,715, 5,856,463, 5,861,288, 5,891,683, 5,891,684, 5,985,621, 5,989,908, 5,998,193, 5,998,203, WO 9858058 by Ludwig and Sproat, WO 9858057 by Ludwig and Sproat, and WO 9718312 by Ludwig and Sproat), hairpin ribozymes
15 (for example, but not limited to, the following United States patents: 5,631,115, 5,646,031, 5,683,902, 5,712,384, 5,856,188, 5,866,701, 5,869,339, and 6,022,962), and tetrahymena ribozymes (for example, but not limited to, the following United States patents: 5,595,873 and 5,652,107). There are also a number of ribozymes that are not found in natural systems, but which have been engineered to catalyze specific reactions de novo (for example, but not
20 limited to, the following United States patents: 5,580,967, 5,688,670, 5,807,718, and 5,910,408). Preferred ribozymes cleave RNA or DNA substrates, and more preferably cleave RNA substrates. Ribozymes typically cleave nucleic acid substrates through recognition and binding of the target substrate with subsequent cleavage. This recognition is often based mostly on canonical or non-canonical base pair interactions. This property makes ribozymes
25 particularly good candidates for target specific cleavage of nucleic acids because recognition of the target substrate is based on the target substrates sequence. Representative examples of how to make and use ribozymes to catalyze a variety of different reactions can be found in the following non-limiting list of United States patents: 5,646,042, 5,693,535, 5,731,295, 5,811,300, 5,837,855, 5,869,253, 5,877,021, 5,877,022, 5,972,699, 5,972,704, 5,989,906, and
30 6,017,756.

Also disclosed are triplex forming functional nucleic acid molecules that interact with the disclosed polynucleotides. Triplex forming functional nucleic acid molecules are molecules that can interact with either double-stranded or single-stranded nucleic acid. When triplex molecules interact with a target region, a structure called a triplex is formed, in which

there are three strands of DNA forming a complex dependant on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind target regions with high affinity and specificity. It is preferred that the triplex forming molecules bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Representative
5 examples of how to make and use triplex forming molecules to bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,176,996, 5,645,985, 5,650,316, 5,683,874, 5,693,773, 5,834,185, 5,869,246, 5,874,566, and 5,962,426.

Also disclosed are external guide sequences that form a complex with the disclosed
10 polynucleotides. External guide sequences (EGSs) are molecules that bind a target nucleic acid molecule forming a complex, and this complex is recognized by RNase P, which cleaves the target molecule. EGSs can be designed to specifically target a RNA molecule of choice. RNase P aids in processing transfer RNA (tRNA) within a cell. Bacterial RNase P can be recruited to cleave virtually any RNA sequence by using an EGS that causes the target
15 RNA:EGS complex to mimic the natural tRNA substrate. (WO 92/03566 by Yale, and Forster and Altman, Science 238:407-409 (1990)).

Similarly, eukaryotic EGS/RNase P-directed cleavage of RNA can be utilized to cleave desired targets within eukarotic cells. (Yuan et al., Proc. Natl. Acad. Sci. USA 89:8006-8010 (1992); WO 93/22434 by Yale; WO 95/24489 by Yale; Yuan and Altman,
20 EMBO J 14:159-168 (1995), and Carrara et al., Proc. Natl. Acad. Sci. (USA) 92:2627-2631 (1995)). Representative examples of how to make and use EGS molecules to facilitate cleavage of a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,168,053, 5,624,824, 5,683,873, 5,728,521, 5,869,248, and 5,877,162.

Also disclosed are polynucleotides that contain peptide nucleic acids (PNAs)
25 compositions. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, Antisense Nucleic Acid Drug Dev. 1997; 7(4) 431-37). PNA is able to be utilized in a number of methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding RNA or
30 DNA sequences and have utilities that are not inherent to RNA or DNA. A review of PNA including methods of making, characteristics of, and methods of using, is provided by Corey (Trends Biotechnol 1997 June; 15(6):224-9). As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of an mRNA sequence based on the disclosed polynucleotides, and such PNA compositions may be used to

regulate, alter, decrease, or reduce the translation of the disclosed polynucleotides transcribed mRNA, and thereby alter the level of the disclosed polynucleotide's activity in a host cell to which such PNA compositions have been administered.

PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen et al., Science Dec. 6, 1991; 254(5037):1497-500; Hanvey et al., Science. Nov. 27, 1992; 258(5087):1481-5; Hyrup and Nielsen, Bioorg Med Chem. 1996 January; 4(1):5-23). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc or Fmoc protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used.

PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, Mass.). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton et al., Bioorg Med Chem. 1995 April; 3(4):437-45). The manual protocol lends itself to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs.

As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, the presence of adjacent purines can lead to deletions of one or more residues in the product. In expectation of this difficulty, it is suggested that, in producing PNAs with adjacent purines, one should repeat the coupling of residues likely to be added inefficiently. This should be followed by the purification of PNAs by reverse-phase high-pressure liquid chromatography, providing yields and purity of product similar to those observed during the synthesis of peptides.

Modifications of PNAs for a given application may be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic acid group to the exposed N-terminal amine. Alternatively, PNAs can be modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry. Several studies have made and utilized modifications of PNAs (for example, Norton et al., Bioorg Med Chem. 1995 April; 3(4):437-45; Petersen et al., J Pept Sci. 1995 May-June; 1(3):175-83; Orum et al., Biotechniques. 1995 September; 19(3):472-80; Footer et al., Biochemistry. Aug. 20, 1996; 35(33): 10673-9; Griffith et al., Nucleic Acids Res. Aug.

11, 1995; 23(15):3003-8; Pardridge et al., Proc Natl Acad Sci USA. Jun. 6, 1995; 92(12):5592-6; Boffa et al., Proc Natl Acad Sci USA. Mar. 14, 1995; 92(6):1901-5; Gambacorti-Passerini et al., Blood. Aug. 15, 1996; 88(4):1411-7; Armitage et al., Proc Natl Acad Sci USA. Nov. 11, 1997; 94(23):12320-5; Seeger et al., Biotechniques. 1997
5 September; 23(3):512-7). U.S. Pat. No. 5,700,922 discusses PNA-DNA-PNA chimeric molecules and their uses in diagnostics, modulating protein in organisms, and treatment of conditions susceptible to therapeutics.

Methods of characterizing the antisense binding properties of PNAs are discussed in Rose (Anal Chem. Dec. 15, 1993; 65(24):3545-9) and Jensen et al. (Biochemistry. Apr. 22,
10 1997; 36(16):5072-7). Rose uses capillary gel electrophoresis to determine binding of PNAs to their complementary oligonucleotide, measuring the relative binding kinetics and stoichiometry. Similar types of measurements were made by Jensen et al. using BIAcore.TM. technology.

Other applications of PNAs that have been described and will be apparent to the skilled
15 artisan include use in DNA strand invasion, antisense inhibition, mutational analysis, enhancers of transcription, nucleic acid purification, isolation of transcriptionally active genes, blocking of transcription factor binding, genome cleavage, biosensors, *in situ* hybridization, and the like.

20 **Vectors and Expression Cassettes**

Adenovirus vectors have been used in eukaryotic gene expression and can be used for gene therapy. Retroviral vectors are also suitable for expressing the polynucleotides of the invention in cells. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by
25 a process of reverse-transcription. The resulting DNA then stably integrates into cellular chromosomes as a pro virus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants.

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is
30 replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed. When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation, for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be

packaged into viral particles, which are then secreted into the culture media. The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus, adeno-associated virus (AAV) and herpesviruses may be employed.

In order to affect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished in vitro, as in laboratory procedures for transforming cells lines, or in vivo or ex vivo, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes and lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In yet another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer in vitro but it may be applied to in vivo use as well.

To prepare expression cassettes, the recombinant DNA sequence or segment may be circular or linear, double-stranded or single-stranded. Generally, the DNA sequence or

segment is in the form of chimeric DNA, such as plasmid DNA or a vector that can also contain coding regions flanked by control sequences that promote the expression of the recombinant DNA present in the resultant transformed cell.

5 A “chimeric” vector or expression cassette, as used herein, means a vector or cassette including nucleic acid sequences from at least two different species, or has a nucleic acid sequence from the same species that is linked or associated in a manner that does not occur in the “native” or wild type of the species.

Aside from recombinant DNA sequences that serve as transcription units for an RNA transcript, or portions thereof, a portion of the recombinant DNA may be untranscribed,
10 serving a regulatory or a structural function. For example, the recombinant DNA may have a promoter that is active in mammalian cells.

Other elements functional in the host cells, such as introns, enhancers, polyadenylation sequences and the like, may also be a part of the recombinant DNA. Such elements may or may not be necessary for the function of the DNA, but may provide
15 improved expression of the DNA by affecting transcription, stability of the miRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the miRNA in the cell.

Control sequences are DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable
20 for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Operably linked nucleic acids are nucleic acids placed in a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked
25 to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked DNA sequences are DNA sequences that are linked are contiguous. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide
30 adaptors or linkers are used in accord with conventional practice.

The recombinant DNA to be introduced into the cells may contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and

used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like.

5 Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. For example,
10 reporter genes include the chloramphenicol acetyl transferase gene (cat) from Tn9 of *E. coli* and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

The general methods for constructing recombinant DNA that can transfect target cells are well known to those skilled in the art, and the same compositions and methods of
15 construction may be utilized to produce the DNA useful herein.

The recombinant DNA can be readily introduced into the host cells, e.g., mammalian, bacterial, yeast or insect cells by transfection with an expression vector composed of DNA encoding the miRNA by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a cell having the recombinant DNA stably integrated
20 into its genome or existing as an episomal element, so that the DNA molecules, or sequences of the present invention are expressed by the host cell. Preferably, the DNA is introduced into host cells via a vector. The host cell is preferably of eukaryotic origin, e.g., plant, mammalian, insect, yeast or fungal sources, but host cells of non-eukaryotic origin may also be employed.

25 Physical methods to introduce a preselected DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. For mammalian gene therapy, as described herein below, it is desirable to use an efficient means of inserting a copy gene into the host genome.
30 Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Patent Nos. 5,350,674 and 5,585,362. In some embodiments,

expression vectors that do not integrate into the genome can also be used to express miR-9 or miR-25 inhibitors.

As discussed herein, a "transfected", "or "transduced" host cell or cell line is one in which the genome has been altered or augmented by the presence of at least one heterologous or recombinant nucleic acid sequence. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence. The transfected DNA can become a chromosomally integrated recombinant DNA sequence, which is composed of sequence encoding the miRNA or the transfected DNA can remain episomal.

To confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

To detect and quantitate RNA produced from introduced recombinant DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the recombinant DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the peptide products of the introduced recombinant DNA sequences or evaluating the phenotypic changes brought about by the expression of the introduced recombinant DNA segment in the host cell.

The instant invention provides a cell expression system for expressing exogenous nucleic acid material in a mammalian recipient. The expression system, also referred to as a "genetically modified cell," comprises a cell and an expression vector for expressing the

exogenous nucleic acid material. The genetically modified cells are suitable for administration to a mammalian recipient, where they replace the endogenous cells of the recipient. Thus, the preferred genetically modified cells are non-immortalized and are non-tumorigenic.

5 According to one embodiment, the cells are transfected or otherwise genetically modified ex vivo. The cells are isolated from a mammal (preferably a human), nucleic acid introduced (i.e., transduced or transfected in vitro) with a vector for expressing a heterologous (e.g., recombinant) gene encoding the therapeutic agent, and then administered to a mammalian recipient for delivery of the therapeutic agent in situ. The mammalian
10 recipient may be a human and the cells to be modified are autologous cells, i.e., the cells are isolated from the mammalian recipient.

According to another embodiment, the cells are transfected or transduced or otherwise genetically modified in vivo. The cells from the mammalian recipient are transduced or transfected in vivo with a vector containing exogenous nucleic acid material for expressing a
15 heterologous (e.g., recombinant) gene encoding a therapeutic agent and the therapeutic agent is delivered in situ.

As used herein, "exogenous nucleic acid material" refers to a nucleic acid or an oligonucleotide, either natural or synthetic, which is not naturally found in the cells; or if it is naturally found in the cells, is modified from its original or native form or is a synthetic
20 version of an RNA naturally found in cells. Thus, "exogenous nucleic acid material" includes, for example, a non-naturally occurring nucleic acid that can be transcribed into an miRNA, as well as a "heterologous gene" (i.e., a gene encoding a protein that is not expressed or is expressed at biologically insignificant levels in a naturally-occurring cell of the same type).

25 **Methods for Introducing the Expression Cassettes of the Invention into Cells**

The inhibitory nucleic acid material (e.g., an expression cassette encoding miR-9 and/or miR-25 inhibitors) can be introduced into the cell ex vivo or in vivo by genetic transfer methods, such as transfection or transduction, to provide a genetically modified cell.
30 Various expression vectors (i.e., vehicles for facilitating delivery of exogenous nucleic acid into a target cell) are known to one of ordinary skill in the art.

As used herein, "transfection of cells" refers to the acquisition by a cell of new nucleic acid material by incorporation of added DNA. Thus, transfection refers to the insertion of nucleic acid into a cell using physical or chemical methods. Several transfection techniques

are known to those of ordinary skill in the art including calcium phosphate DNA co-precipitation, DEAE-dextran, electroporation, cationic liposome-mediated transfection, lipid nanoparticle mediated transfection, tungsten particle-facilitated microparticle bombardment, and strontium phosphate DNA co-precipitation.

5 In contrast, "transduction of cells" refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. A RNA virus (i.e., a retrovirus) for transferring a nucleic acid into a cell is referred to herein as a transducing chimeric retrovirus. Exogenous nucleic acid material contained within the retrovirus is incorporated into the genome of the transduced cell. A cell that has been transduced with a chimeric DNA virus (e.g., an
10 adenovirus carrying a cDNA encoding a therapeutic agent), will not have the exogenous nucleic acid material incorporated into its genome but will be capable of expressing the exogenous nucleic acid material that is retained extrachromosomally within the cell.

The exogenous nucleic acid material can include the nucleic acid encoding the miRNA together with a promoter to control transcription. The promoter characteristically
15 has a specific nucleotide sequence necessary to initiate transcription. The exogenous nucleic acid material may further include additional sequences (i.e., enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any non-translated DNA sequence that works with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The exogenous nucleic acid material may
20 be introduced into the cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. An expression vector can include an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and regulatable promoters.

25 Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a nucleic acid sequence under the control of a constitutive promoter is expressed under all conditions of cell growth. Constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR),
30 adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the beta $\square$ -actin promoter, and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eukaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRs) of Moloney

Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others.

Nucleic acid sequences that are under the control of regulatable promoters are expressed only or to a greater or lesser degree in the presence of an inducing or repressing agent, (e.g., transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Regulatable promoters include responsive elements (REs) that stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid, cyclic AMP, and tetracycline and doxycycline. Promoters containing a particular RE can be chosen in order to obtain a regulatable response and in some cases, the RE itself may be attached to a different promoter, thereby conferring regulatability to the encoded nucleic acid sequence. Thus, by selecting the appropriate promoter (constitutive versus regulatable; strong versus weak), it is possible to control both the existence and level of expression of a nucleic acid sequence in the genetically modified cell. If the nucleic acid sequence is under the control of an regulatable promoter, delivery of the therapeutic agent in situ is triggered by exposing the genetically modified cell in situ to conditions for permitting transcription of the nucleic acid sequence, e.g., by intraperitoneal injection of specific inducers of the regulatable promoters which control transcription of the agent. For example, in situ expression of a nucleic acid sequence under the control of the metallothionein promoter in genetically modified cells is enhanced by contacting the genetically modified cells with a solution containing the appropriate (i.e., inducing) metal ions in situ.

Accordingly, the amount of miR-9 and/or miR-25 inhibitor generated in situ is regulated by controlling such factors as the nature of the promoter used to direct transcription of the nucleic acid sequence, (i.e., whether the promoter is constitutive or regulatable, strong or weak) and the number of copies of the exogenous nucleic acid sequences encoding miR-9 or miR-25 that are in the cell.

In addition to at least one promoter and at least one heterologous nucleic acid sequence encoding the miR-9 and/or miR-25 inhibitor, the expression vector may include a selection gene, for example, a neomycin resistance gene, for facilitating selection of cells that have been transfected or transduced with the expression vector.

Cells can also be transfected with two or more expression vectors, at least one vector containing the nucleic acid sequence(s) encoding the miR-9 and/or miR-25 inhibitor, the other vector containing a selection gene. The selection of a suitable promoter, enhancer,

selection gene, and/or signal sequence is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

Delivery Vehicles for the Expression Cassettes of the Invention

5 Delivery of compounds into tissues can be limited by the size and biochemical properties of the compounds. The selection and optimization of a particular expression vector for expressing miR-9 and/or miR-25 inhibitor in a cell can be accomplished by obtaining the nucleic acid sequence of the miR-9 and/or miR-25 inhibitor, possibly with one or more appropriate control regions (e.g., promoter, insertion sequence); preparing a vector
10 construct comprising the vector into which is inserted the nucleic acid sequence encoding the miR-9 and/or miR-25 inhibitor; transfecting or transducing cultured cells in vitro with the vector construct; and determining whether the miR-9 and/or miR-25 inhibitor is present in the cultured cells.

 Vectors for cell therapy include viruses, such as replication-deficient viruses
15 (described in detail herein). Exemplary viral vectors are derived from Harvey Sarcoma virus, ROUS Sarcoma virus, (MPSV), Moloney murine leukemia virus and DNA viruses (e.g., adenovirus).

 Replication-deficient retroviruses are capable of directing synthesis of all virion proteins, but are incapable of making infectious particles. Accordingly, these genetically
20 altered retroviral expression vectors have general utility for high-efficiency transduction of nucleic acid sequences in cultured cells, and specific utility for use in the method of the present invention. Such retroviruses further have utility for the efficient transduction of nucleic acid sequences into cells in vivo. Retroviruses have been used extensively for transferring nucleic acid material into cells. Protocols for producing replication-deficient
25 retroviruses (including the steps of incorporation of exogenous nucleic acid material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with the viral particles) are well known in the art.

 An advantage of using retroviruses for therapy is that the viruses insert the nucleic
30 acid sequence encoding the miR-9 and/or miR-25 inhibitor into the host cell genome, thereby permitting the nucleic acid sequence encoding the miR-9 and/or miR-25 inhibitor to be passed on to the progeny of the cell when it divides. Promoter sequences in the LTR region have can enhance expression of an inserted coding sequence in a variety of cell types. Some disadvantages of using a retrovirus expression vector are (1) insertional mutagenesis, i.e., the

insertion of the nucleic acid sequence encoding the miR-9 and/or miR-25 inhibitor into an undesirable position in the target cell genome which, for example, leads to unregulated cell growth and (2) the need for target cell proliferation in order for the nucleic acid sequence encoding the miR-9 and/or miR-25 inhibitor carried by the vector to be integrated into the target genome.

Another viral candidate useful as an expression vector for transformation of cells is the adenovirus, a double-stranded DNA virus. The adenovirus is infective in a wide range of cell types, including, for example, muscle and endothelial cells.

Adenoviruses (Ad) are double-stranded linear DNA viruses with a 36 kb genome. Several features of adenovirus have made them useful as transgene delivery vehicles for therapeutic applications, such as facilitating in vivo gene delivery. Recombinant adenovirus vectors have been shown to be capable of efficient in situ gene transfer to parenchymal cells of various organs, including the lung, brain, pancreas, gallbladder, and liver. This has allowed the use of these vectors in methods for treating inherited genetic diseases, such as cystic fibrosis, where vectors may be delivered to a target organ. In addition, the ability of the adenovirus vector to accomplish in situ tumor transduction has allowed the development of a variety of anticancer gene therapy methods for non-disseminated disease. In these methods, vector containment favors tumor cell-specific transduction.

Like the retrovirus, the adenovirus genome is adaptable for use as an expression vector for gene therapy, i.e., by removing the genetic information that controls production of the virus itself. Because the adenovirus functions in an extrachromosomal fashion, the recombinant adenovirus does not have the theoretical problem of insertional mutagenesis.

Several approaches traditionally have been used to generate the recombinant adenoviruses. One approach involves direct ligation of restriction endonuclease fragments containing a nucleic acid sequence of interest to portions of the adenoviral genome. Alternatively, the nucleic acid sequence of interest may be inserted into a defective adenovirus by homologous recombination results. The desired recombinants are identified by screening individual plaques generated in a lawn of complementation cells.

Most adenovirus vectors are based on the adenovirus type 5 (Ad5) backbone in which an expression cassette containing the nucleic acid sequence of interest has been introduced in place of the early region 1 (E1) or early region 3 (E3). Viruses in which E1 has been deleted are defective for replication and are propagated in human complementation cells (e.g., 293 or 911 cells), which supply the missing gene E1 and pIX in trans.

Administration of miR-9 and/or miR-25 inhibitors is generally accomplished by transfection of synthetic miR-9 and/or miR-25 inhibitors, in vitro synthesized RNAs, or plasmids expressing miR-9 and/or miR-25 inhibitors. Recombinant adenovirus, adeno-associated virus (AAV) and feline immunodeficiency virus (FIV) can be used to deliver genes in vitro and in vivo. Each has its own advantages and disadvantages. Adenoviruses are double stranded DNA viruses with large genomes (36 kb) and have been engineered by my laboratory and others to accommodate expression cassettes in distinct regions.

Adeno-associated viruses have encapsidated genomes, similar to Ad, but are smaller in size and packaging capacity (~30 nm vs. ~100 nm; packaging limit of ~4.5 kb). AAV contain single stranded DNA genomes of the + or the - strand. Eight serotypes of AAV (1-8) have been studied extensively.

Adeno associated virus (AAV) is a small nonpathogenic virus of the parvoviridae family. AAV is distinct from the other members of this family by its dependence upon a helper virus for replication. In the absence of a helper virus, AAV may integrate in a locus specific manner into the q arm of chromosome 19. The approximately 5 kb genome of AAV consists of one segment of single stranded DNA of either plus or minus polarity. The ends of the genome are short inverted terminal repeats which can fold into hairpin structures and serve as the origin of viral DNA replication. Physically, the parvovirus virion is non-enveloped and its icosahedral capsid is approximately 20 nm in diameter.

Further provided by this invention are chimeric viruses where AAV can be combined with herpes virus, herpes virus amplicons, baculovirus or other viruses to achieve a desired tropism associated with another virus. For example, the AAV4 ITRs could be inserted in the herpes virus and cells could be infected. Post-infection, the ITRs of AAV4 could be acted on by AAV4 rep provided in the system or in a separate vehicle to rescue AAV4 from the genome. Therefore, the cellular tropism of the herpes simplex virus can be combined with AAV4 rep mediated targeted integration. Other viruses that could be utilized to construct chimeric viruses include lentivirus, retrovirus, pseudotyped retroviral vectors, and adenoviral vectors.

Also provided by this invention are variant AAV vectors. For example, the sequence of a native AAV, such as AAV5, can be modified at individual nucleotides. The present invention includes native and mutant AAV vectors. The present invention further includes all AAV serotypes.

FIV is an enveloped virus with a strong safety profile in humans; individuals bitten or scratched by FIV-infected cats do not seroconvert and have not been reported to show any

signs of disease. Like AAV, FIV provides lasting transgene expression in mouse and nonhuman primate neurons, and transduction can be directed to different cell types by pseudotyping, the process of exchanging the virus's native envelope for an envelope from another virus.

5 Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable viral expression vectors are available for transferring exogenous nucleic acid material into cells. The selection of an appropriate expression vector to express a therapeutic agent for a particular condition amenable to gene silencing therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of
10 one of ordinary skill in the art without the need for undue experimentation.

 In another embodiment, the expression vector is in the form of a plasmid, which is transferred into the target cells by one of a variety of methods: physical (e.g., microinjection, electroporation, scrape loading, microparticle bombardment) or by cellular uptake as a chemical complex (e.g., calcium or strontium co-precipitation, complexation with lipid,
15 complexation with ligand). Several commercial products are available for cationic liposome complexation including Lipofectin™ (Gibco-BRL, Gaithersburg, Md.) and Transfectam™ (Promega®, Madison, Wis.). However, the efficiency of transfection by these methods is highly dependent on the nature of the target cell and accordingly, the conditions for optimal transfection of nucleic acids into cells using the herein-mentioned procedures must be
20 optimized. Such optimization is within the scope of one of ordinary skill in the art without the need for undue experimentation.

 In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers
25 separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Also contemplated are lipofectamine-DNA complexes. Liposome-mediated nucleic acid delivery and expression of foreign DNA in vitro has been very successful. Where a
30 bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

 In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, lactosyl-ceramide, a galactose-terminal asialganglioside, was incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it

is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type by any number of receptor-ligand systems with or without liposomes. For example, mannose can be used to target the mannose receptor on liver cells.

In a particular example, the miR-9 and/or miR-25 inhibitors may be administered in combination with a cationic lipid. Examples of cationic lipids include, but are not limited to, lipofectin, DOTMA, DOPE, and DOTAP. The publication of WO/0071096, which is specifically incorporated by reference, describes different formulations, such as a DOTAP: cholesterol or cholesterol derivative formulation that can effectively be used for gene therapy. Other disclosures also discuss different lipid or liposomal formulations including nanoparticles and methods of administration; these include, but are not limited to, U.S. Patent Publication 20030203865, 20020150626, 20030032615, and 20040048787, which are specifically incorporated by reference to the extent they disclose formulations and other related aspects of administration and delivery of nucleic acids. Methods used for forming particles are also disclosed in U.S. Patents 5,844,107, 5,877,302, 6,008,336, 6,077,835, 5,972,901, 6,200,801, and 5,972,900, which are incorporated by reference.

In certain embodiments, gene transfer may more easily be performed under ex vivo conditions. Ex vivo gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells in vitro, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

Solid Substrate

In certain embodiments, the therapeutic agent comprising a miR-9 inhibitor and/or miR-25 inhibitor may be placed (e.g., coated) onto various solid substrates, such as a catheter (e.g., a peripheral intravenous catheter, a central venous catheter, or a catheter hub, or a catheter port) or a non-degradable implant (e.g., a prosthetic heart valve). In particular, the coating may be placed on vascular catheters that are typically used for long periods of time, such as with dialysis and cancer patients. In certain embodiments, the solid substrate is a component of silica, cellulose, cellulose acetate, nitrocellulose, nylon, polyester, polyethersulfone, polyolefin, or polyvinylidene fluoride, or combinations thereof. In certain embodiments, the solid substrate is a stent or other medical device.

Treatment of Disease

In vascular disease, there are difficulties with vascular remodeling because the smooth muscle cells in the blood vessels become activated. When activated, the cells de-differentiate, migrate, and proliferate in the blood vessel wall. miRNA-9 and miRNA-25
5 regulate the expression of NOX4, the enzyme responsible for cell differentiation. Thus, means of silencing the appropriate miRNA can inhibit or reduce the signs and symptoms of, for example, vascular disease.

Smooth muscle cells (SMC) switch from a differentiated (contractile) to a dedifferentiated (synthetic) phenotype when subjected to appropriate environmental cues,
10 allowing them to proliferate and migrate. An example of SMC plasticity occurs during development of blood vessels. The switching from a differentiated smooth muscle to a dedifferentiated smooth muscle phenotype contributes to the development of vascular disease (atherosclerosis, bypass graft stenosis, angioplasty/stent restenosis) and some cancers (gastrointestinal, bladder, uterine). In addition, tumor growth is in general dependent on
15 development of new blood vessels (neovascularization). MicroRNAs are small, noncoding RNAs that regulate gene expression. Two different miRNAs, miR9 and miR25, have been identified that are important in the dedifferentiation of smooth muscle cells. By administering an inhibitor of miR9 or miR25, the dedifferentiation of smooth muscle cells is prevented, which is useful in preventing human disease.

20 In certain embodiments, the present invention provides a method to modulate smooth muscle differentiation. Inhibitors of microRNA-9 and/or microRNA-25 are used to prevent the silencing of genes important in smooth muscle differentiation. In certain embodiments, the present invention treats vascular diseases (atherosclerosis, bypass graft stenosis, angioplasty/stent restenosis) and some cancers (gastrointestinal, bladder, uterine).

25 Cellular levels of miR9 are increased in dedifferentiated smooth muscle cells from the blood vessel and in some smooth muscle cell cancers. In cell culture and in a mouse model of vascular injury, an inhibitor of miR9 prevents smooth muscle cell dedifferentiation, attenuates the migration of cells to growth factors, and reduces the intima formation in response to vascular injury. Expression of miR9 is increased in endometrial cancer from
30 patient samples as per a database. An inhibitor of miR25 causes the decrease in miR9 levels in endometrial cancer cell lines.

Disclosed herein are methods to prevent, inhibit or reduce the risk of a disease or a condition that can be the result of an injury to the vascular or circulatory system. Such disease or condition can be caused by a medical procedure or medical treatment. For

example, in response to an injury, a normal blood vessel initiates multiple intracellular signaling pathways that allow the smooth muscle cell to de-differentiate from its quiescent and contractile phenotype. These de-differentiated synthetic smooth muscle cells degrade the extracellular matrix, migrate to the sub-endothelial space, and proliferate. This process is activated under multiple clinical scenarios and can result in dysregulated smooth muscle cell growth with occlusion of the vascular lumen. For instance, the standard treatment of an arterial stenosis is balloon angioplasty with or without the deployment of a stent. The resulting trauma to the vascular wall can trigger the activation of smooth muscle cells such that they cause restenosis within months. Similarly, when veins are harvested for purposes of bypass grafting, the ensuing trauma and re-implantation can activate vascular cells causing development of accelerated graft disease. In both of these situations, the disease that develops does not represent the return of the original disease but instead, the development of disease secondary to activation of the smooth muscle cells in the vessel wall. The miRNAs miR-9 and miR-25 are expected to be increased (see, for example, FIG. 19), and subsequently cause the silencing of multiple genes (including Nox4 and myocardin) involved in smooth muscle cell differentiation. Accordingly, in an aspect, administration of a miR-9 and/or miR-25 inhibitor can protect, prevent or reduce the risk of arterial restenosis or accelerated venous bypass disease from the activation of smooth muscle cells. Diverse conditions that cause vascular injury and the subsequent development of hyperproliferative disease can involve the expression and action of miR-9 and mR-25. Examples of these conditions include but are not limited to (1) vascular balloon angioplasty; (2) deployment of arterial stent; (3) artero-venous fistula creation for hemodialysis; (4) pulmonary toxins, hyperoxia/hypoxia involved in the development of pulmonary hypertension; and (5) organ transplant vasculopathy.

Methods of Treatment

Certain embodiments of the invention provide a method of treating vascular disease in a mammal (e.g., a human), comprising administering an effective amount of a miR-9 and/or miR-25 inhibitor to the mammal.

Certain embodiments of the invention provide a miR-9 and/or miR-25 inhibitor for use in treating vascular disease in a mammal (e.g., a human).

Certain embodiments of the invention provide the use of a miR-9 and/or miR-25 inhibitor to prepare a medicament for treating vascular disease in a mammal (e.g., a human).

Certain embodiments of the invention provide a miR-9 and/or miR-25 inhibitor for use in medical therapy.

As used herein, “treating” or “treatment” refers to reversing, alleviating, delaying the onset of, inhibiting the progress of, and/or preventing a disease or disorder, or one or more symptoms thereof, to which the term is applied in a subject. In some embodiments, treatment may be applied after one or more symptoms have developed. In other embodiments, treatment may be administered in the absence of symptoms. For example, treatment may be administered prior to symptoms (e.g., in light of a history of symptoms and/or one or more other susceptibility factors), or after symptoms have resolved, for example to prevent or delay their reoccurrence.

Disclosed herein are methods of preventing or reducing the risk of graft disease in a subject. Also, disclosed herein are methods of preventing or reducing the risk of restenosis in a subject. Further, disclosed herein, are methods of preventing or reducing the risk of arterial restenosis or accelerated venous bypass disease in a subject. In an aspect, the method comprises administering to the subject or patient an effective amount of a therapeutic agent. In an aspect, the therapeutic agent comprises a miR-9 inhibitor and a miR-25 inhibitor. In some aspects, the therapeutic agent is administered to the subject prior to, during or after a medical procedure. Examples of medical procedures include but are not limited to vascular balloon angioplasty, deployment of an arterial stent, artero-venous fistula creation for hemodialysis, exposure or contact to one or more pulmonary toxins, hyperoxia/hypoxia involved in the development of pulmonary hypertension or organ transplant vasculopathy.

As used herein, the term “prior to” can mean seconds, minutes or hours before said administration of the therapeutic agent as described herein. For example, the therapeutic agent can be administered 48, 36, 24, 18, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 hour prior to the start of, for example, a medical procedure, or 50, 40, 30, 20, 10, 5, or 1 minute prior to treatment for example, a medical procedure, or any amount in between. The therapeutic agent as described herein can also be administered after the medical procedure has begun. The therapeutic agent can be administered 24, 18, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 hours after the medical procedure started, or 50, 40, 30, 20, 10, 5, or 1 minute after the medical procedure has begun, or any amount in between these times. The therapeutic agent as described herein can also be administered before, during, or after the exposure to one or more pulmonary toxins, as discussed herein.

As used herein, a miR-9 and/or miR-25 inhibitor refers to a molecule capable of reducing the expression and/or function of miR-9 and/or miR-25 in a cell. In certain embodiments, the miR-9 and/or miR-25 inhibitor reduces miR-9 and/or miR-25 expression. In certain embodiments, the miR-9 and/or miR-25 inhibitor reduces miR-9 and/or miR-25

function. In certain embodiments, the expression and/or function of miR-9 and/or miR-25 in a cell is reduced by about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, as compared to the expression or function of a miR-9 and/or miR-25 in a cell (e.g., in a mammal) that has not been administered/contacted with the inhibitor. miR-9 and/or miR-25 expression and/or function may be determined using assays known in the art. For example, miRNA expression may be measured using an activatable sensor oligonucleotide, RT-PCR, Real-Time qRT-PCR, or northern blotting, or in-situ hybridization, whereas miRNA function may be measured by examining the expression of miRNA targets.

Inhibitors may interact directly or indirectly with miR-9 and/or miR-25 and include, for example, oligonucleotides, including miRNA sponges (Ebert et al., Nature Methods, 4, 721-726 (2007)), and small molecules.

In certain embodiments, the administration of the miR-9 and/or miR-25 inhibitor causes increased or maintained Nox4 NADPH oxidase expression in a cell in the mammal, as compared to Nox4 NADPH oxidase expression in a cell in a mammal that was not administered the miR-9 and/or miR-25 inhibitor. In certain embodiments, Nox4 NADPH oxidase expression is increased by about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, as compared to Nox4 NADPH oxidase expression in a cell in a mammal that was not administered the miR-9 and/or miR-25 inhibitor. In certain embodiments, Nox4 NADPH oxidase expression is increased or unchanged in vascular smooth muscle cells.

In certain embodiments, the administration of the miR-9 and/or miR-25 inhibitor causes an increase in Nox4 NADPH oxidase expression in a cell in the mammal, as compared to Nox4 NADPH oxidase expression in a cell in a mammal that was not administered the miR-9 and/or miR-25 inhibitor. In certain embodiments, Nox4 NADPH oxidase expression is increased by about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, as compared to eNOS expression in a cell in a mammal that was not administered the miR-9 and/or miR-25 inhibitor. In certain embodiments, Nox4 NADPH oxidase expression is increased or unchanged in vascular smooth muscle cells.

As used herein, the term "unchanged" can mean that the expression level of the gene or protein in a cell administered the treatment disclosed herein, for example, is not significantly different from the expression level in a cell not administered the treatment.

As used herein, Nox4 NADPH oxidase expression may be used to refer to expression of the RNA transcript or the translated protein. Expression may be determined using assays

known in the art, for example, RT-PCR, Real-Time qRT-PCR, Western blot, immunofluorescence, or immunocytochemistry.

In an aspect, the administration of the miR-9 and miR-25 inhibitor increases or has no effect on ROS levels when compared to ROS levels in a cell in subject that was not administered the therapeutic agent.

In an aspect, the administration of the miR-9 and miR-25 inhibitor increases or has no effect on mRNA expression of myocardin when compared to mRNA expression of myocardin in a cell in subject that was not administered the therapeutic agent.

In an aspect, the administration of the miR-9 and miR-25 inhibitor reduces or prevents cell migration when compared to cell migration in subject that was not administered the therapeutic agent.

In an aspect, the administration of the miR-9 and miR-25 inhibitor increases or has no effect on serum response factor levels when compared to levels of serum response factor in a cell in subject that was not administered the therapeutic agent.

Disclosed herein are methods of improving contractility of a vascular smooth muscle cell that expresses miR-9 or miR-25. In an aspect, the method comprises contacting the cell in vivo or in vitro with a miR-9 inhibitor and miR-25 inhibitor as described herein.

In certain embodiments, the miR-9 inhibitor comprises an oligonucleotide (e.g., a deoxyribooligonucleotide or a ribooligonucleotide) comprising 4 to 7 nucleotides (e.g., 4, 5, 6, or 7 nucleotides) that are complementary to a seed sequence of miR-9 (3'- UGGUUUC -'5 (SEQ ID NO: 9). Thus, the sequence of the inhibitor comprises 4 to 7 nucleotides (e.g., 4, 5, 6, or 7 nucleotides) of the sequence UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO: 2).

In certain embodiments, the miR-9 inhibitor comprises an oligonucleotide (e.g., a deoxyribooligonucleotide or a ribooligonucleotide) comprising a sequence having at least about 70% complementarity with a seed sequence of miR-9 3'- UGGUUUC -'5 (SEQ ID NO: 9). In certain embodiments, the oligonucleotide comprises a sequence having at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% complementarity to a seed sequence of miR-9.

In certain embodiments, the miR-25 inhibitor comprises an oligonucleotide (e.g., a deoxyribooligonucleotide or a ribooligonucleotide) comprising 4 to 7 nucleotides (e.g., 4, 5, 6, or 7 nucleotides) that are complementary to a seed sequence of miR-25 3'-CACGUUA-'5

(SEQ ID NO: 7). Thus, the sequence of the inhibitor comprises 4 to 7 nucleotides (e.g., 4, 5, 6 or 7 nucleotides) of the sequence CAUUGCACUUGUCUCGGUCUGA (SEQ ID NO: 8).

In certain embodiments, the miR-25 inhibitor comprises an oligonucleotide (e.g., a deoxyribooligonucleotide or a ribooligonucleotide) comprising a sequence having at least about 70% complementarity with a seed sequence of miR-25 3'-CACGUUA-5' (SEQ ID NO: 8). In certain embodiments, the oligonucleotide comprises a sequence having at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% complementarity to a seed sequence of miR-25.

The present invention provides modified and unmodified nucleic acids (e.g., oligonucleotides). Accordingly, in certain embodiments, an oligonucleotide may contain one or more chemical modifications to the nucleotides themselves (e.g., locked nucleic acids), the nucleoside linkage (e.g., phosphorothioate, methyl phosphonate, phosphoramidate, or a combination thereof), or to the sugar moiety.

In certain embodiments, the miR-9 and/or miR-25 inhibitor comprises a miRNA sponge. As used herein a "miRNA sponge" refers to a RNA transcript containing multiple, tandem binding sites to a microRNA of interest (e.g., miR-9 and/or miR-25); sponges specifically inhibit microRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire microRNA seed family (Ebert et al., Nature Methods, 4, 721-726 (2007)). Sponges generally consist of multiple binding sites to the miRs (ranging from, but not limited to, 3-10 nucleotides). Another way to inhibit miR function involves using TuD (Tough Decoys) RNAs. TuDs have miRs binding site (s) in the single stranded regions of short stem-loops. In certain embodiments, an oligonucleotide or miRNA sponge described herein is chemically synthesized. In certain embodiments, an oligonucleotide or miRNA sponge described herein is expressed in vivo from a vector. Accordingly, certain embodiments of the invention provide a method of treating endothelial dysfunction, cardiovascular disease and/or atherosclerosis in a mammal, comprising administering a vector comprising an expression cassette comprising a promoter operably linked to a miR-9 and/or miR-25 inhibitor (e.g., an oligonucleotide or miRNA sponge as described herein), to the mammal. In certain embodiments, the vector is a plasmid. In certain embodiments, vector is a viral vector.

In certain embodiments, the miR-9 and/or miR-25 inhibitor comprises a small molecule inhibitor. In certain embodiments the small molecule inhibits binding between

miR-9 and/or miR-25 and a target gene (e.g., Nox4). In certain embodiments, the small molecule inhibitor is an inhibitor identified using a method described herein.

Certain embodiments of the invention provide a method of decreasing dysfunction in an vascular smooth muscle cell that expresses miR-9 and/or miR-25, comprising contacting
5 the cell in vivo or in vitro with a miR-9 and/or miR-25 inhibitor.

Certain embodiments of the invention provide a method of decreasing Nox4 NADPH oxidase expression in an endothelial cell that expresses miR-9 and/or miR-25, comprising contacting the cell in vivo or in vitro with a miR-9 and/or miR-25 inhibitor. In certain
embodiments, the cell is vascular smooth muscle cell.

10

Administration

The miR-9 and/or miR-25 inhibitors described herein can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, i.e., orally or parenterally,
15 by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the inhibitors may be systemically administered, e.g., intravenously, in combination with a pharmaceutically acceptable vehicle such as an inert diluent. The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally
20 mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, 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 an aspect, the therapeutic agents disclosed herein can be locally administered. For
25 example, the therapeutic agents can be delivered to the site or almost directly to the site of action. For instance, the therapeutic agents disclosed herein can be delivered via a drug eluting arterial stent.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which
30 are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid

polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about
5 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, buffers 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.

10 Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus
15 any additional desired ingredient present in the previously sterile-filtered solutions.

Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant
20 liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Useful dosages of the inhibitors can be determined by comparing their in vitro activity, and in vivo activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S.
25 Pat. No. 4,938,949.

The amount of the inhibitor, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

30 The inhibitor may be conveniently formulated in unit dosage form; in one embodiment, the invention provides a composition comprising a miR-9 and/or miR-25 inhibitor formulated in such a unit dosage form. Alternatively, the miR-9 and/or miR-25 inhibitor can be formulated separately. As such, the miR-9 and/or miR-25 inhibitor can be administered simultaneously or sequentially in any order.

The therapeutic agents described herein can be administered in "therapeutically effective amounts." As used herein, the phrase "therapeutically effective amount" refers to an amount that is expected to be effective, either upon a single or multiple dose administration to a patient, in preventing, curing, reducing the severity of, reducing the risk of, inhibiting, or ameliorating one or more symptoms of a condition or disease described herein. A therapeutically effective amount is an amount that brings about or is expected to bring about a clinically beneficial outcome.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

In certain embodiments, the miR-9 and/or miR-25 inhibitor is an oligonucleotide as described herein (e.g., an antisense oligonucleotide). In certain embodiments, the oligonucleotide is administered via intravenous injection. In certain embodiments, the oligonucleotide is administered weekly, e.g., for 2, 3, 4, 5, 6, 7, 8, 9 or more weeks. As discussed above, useful dosages of the inhibitors depend on a number of factors and may be determined by one skilled in the art. However, in certain embodiments, a suitable dose will be in the range of from about 0.5 to about 100 mg/kg, e.g., from about 1 to about 75 mg/kg of body weight, such as 1 to about 50 mg per kilogram body weight of the recipient, 1 to 10 mg/kg, or 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mg/kg administered weekly. In certain embodiments, the oligonucleotide may be administered in combination with liposomes/microvesicles/cholesterol, which may improve delivery, stability and/or distribution of the oligonucleotide, and thereby its therapeutic benefits. Additionally, as discussed herein, an oligonucleotide described herein may be chemically modified, which may improve its delivery, stability and/or distribution, and thereby its therapeutic benefits. Accordingly, in certain embodiments, the oligonucleotide may be chemically modified, e.g., the backbone may be chemically modified.

The inhibitors can also be administered in combination with other therapeutic agents, for example, other agents that are useful for the treatment of vascular disease. Accordingly, in one embodiment the invention also provides a composition comprising a miR-9 and/or miR-25 inhibitor, or a pharmaceutically acceptable salt thereof, at least one other therapeutic agent, and a pharmaceutically acceptable diluent or carrier. The invention also provides a kit comprising a miR-9 and/or miR-25 inhibitor, or a pharmaceutically acceptable salt thereof, at

least one other therapeutic agent, packaging material, and instructions for administering the miR-9 and/or miR-25 inhibitor or the pharmaceutically acceptable salt thereof and the other therapeutic agent or agents to a mammal to treat of cardiovascular disease, endothelial dysfunction and/or atherosclerosis.

5

General Terminology and Methods

The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,”
10 “having,” “including,” and “containing” are to be construed as open-ended terms (*i.e.*, meaning “including, but not limited to”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

15 All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating
20 any non-claimed element as essential to the practice of the invention.

The term “gene” is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, “gene” refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory
25 sequences. “Genes” also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. “Genes” can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. An “allele” is one of several alternative forms of a gene occupying a given locus on a chromosome.

30 The term “endogenous gene” refers to a native gene in its natural location in the genome of an organism.

A “transgene” refers to a gene that has been introduced into the genome by transformation. Transgenes include, for example, DNA that is either heterologous or

homologous to the DNA of a particular cell to be transformed. Additionally, transgenes may include native genes inserted into a non-native organism, or chimeric genes.

The term “nucleic acid” refers to deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base that is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides.

Unless otherwise indicated, a particular nucleic acid sequence also encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. A “nucleic acid fragment” is a portion of a given nucleic acid molecule.

A “nucleotide sequence” is a polymer of DNA or RNA that can be single-stranded or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

The terms “nucleic acid,” “nucleic acid molecule,” “nucleic acid fragment,” “nucleic acid sequence or segment,” or “polynucleotide” are used interchangeably and may also be used interchangeably with gene, cDNA, DNA and RNA encoded by a gene.

The terms “isolated and/or purified” refer to in vitro isolation of a nucleic acid, e.g., a DNA or RNA molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. The RNA or DNA is “isolated” in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is preferably substantially free of any other mammalian RNA or DNA. The phrase “free from at least one contaminating source nucleic acid with which it is normally associated” includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell, e.g., in a vector or plasmid.

“Naturally occurring,” “native,” or “wild-type” is used to describe an object that can be found in nature as distinct from being artificially produced. For example, a protein or nucleotide sequence present in an organism (including a virus), which can be isolated from a

source in nature and that has not been intentionally modified by a person in the laboratory, is naturally occurring.

A “variant” of a molecule is a sequence that is substantially similar to the sequence of the native molecule. Naturally occurring allelic variants such as these can be identified with the use of molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis. Generally, nucleotide sequence variants of the invention will have at least 40%, 50%, 60%, to 70%, e.g., 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81%-84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98%, sequence identity to the native (endogenous) nucleotide sequence.

As used herein, the term “recombinant nucleic acid”, e.g., “recombinant DNA/RNA sequence or segment” refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate cellular source, that may be subsequently chemically altered in vitro, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been transformed with exogenous DNA. An example of preselected DNA “derived” from a source would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA “isolated” from a source would be a useful DNA sequence that is excised or removed from a source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. “Recombinant DNA” includes completely synthetic DNA sequences, semi synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

The term “RNA transcript” or “transcript” refers to the product resulting from RNA polymerase catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complimentary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. “Messenger RNA” (mRNA) refers to the RNA that is without introns and that can be translated into protein by the cell.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) “reference sequence,” (b) “comparison window,”

(c) “sequence identity,” (d) “percentage of sequence identity,” (e) “substantial identity,” (f) “complementary,” (g) “percent complementarity” and (h) “substantial complementarity.”

(a) As used herein, “reference sequence” is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete
5 cDNA or gene sequence.

(b) As used herein, “comparison window” makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the
10 reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the
15 number of matches.

Methods of alignment of sequences for comparison are well-known in the art. Thus, the determination of percent identity or percent complementarity between any two sequences can be accomplished using a mathematical algorithm.

Computer implementations of these mathematical algorithms can be utilized for
20 comparison of sequences to determine sequence identity, including complementarity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science
25 Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which
30 either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores

are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0).

For amino acid sequences, a scoring matrix is used to calculate the cumulative score.

Extension of the word hits in each direction are halted when the cumulative alignment score

5 falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm also

performs a statistical analysis of the similarity between two sequences. One measure of

10 similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less
15 than about 0.01, and most preferably less than about 0.001.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g.

20 BLASTN for nucleotide sequences) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. Alignment may also be performed manually by inspection.

For sequence comparison, typically one sequence acts as a reference sequence to

25 which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

30 (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid sequences makes reference to a specified percentage of nucleotides in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window, as measured by sequence comparison algorithms or by visual inspection.

(d) As used herein, “percentage of sequence identity” means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e) The term “substantial identity” of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters.

(f) “Complementary” as used herein refers to the broad concept of subunit sequence complementarity between two nucleic acids, e.g., an antisense oligonucleotide and a miRNA. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position.

(g) As used herein, “percent complementarity” means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of corresponding positions in each of the molecules that are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs), dividing the number of positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of complementarity.

(h) Two nucleic acids are “substantially complementary” to each other when at least about 50%, at least about 60%, at least about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of corresponding positions in each of the nucleic acid

molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs).

Another indication that nucleotide sequences are substantially identical/complementary is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein.

The phrase “hybridizing specifically to” refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

“Bind(s) substantially” refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

“Stringent hybridization conditions” and “stringent hybridization wash conditions” in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation : $T_m = 81.5^{\circ}\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal

melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently
5 described. If the desired degree of mismatching results in a T of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH.

10 An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook and Russell 2001, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. For short nucleic acid sequences (e.g., about 10 to 50 nucleotides), stringent conditions
15 typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates
20 detection of a specific hybridization. Very stringent conditions are selected to be equal to the T_m for a particular nucleic acid molecule.

Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50%
25 formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1M NaCl, 1% SDS (sodium dodecyl sulfate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45%
30 formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C.

General methods for constructing recombinant DNA that can be introduced into target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein.

In another embodiment, the miR-9 and/or miR-25 inhibitor may be expressed in vivo from a vector. A "vector" is a composition of matter that can be used to deliver a nucleic acid of interest to the interior of a cell. A "vector" is defined to include, inter alia, any viral vector, as well as any plasmid, cosmid, phage or binary vector in double or single stranded
5 linear or circular form that may or may not be self-transmissible or mobilizable, and that can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g., autonomous replicating plasmid with an origin of replication). Vectors include linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an
10 autonomously replicating plasmid or a virus. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like. An expression construct can be replicated in a living cell, or it can be made synthetically. For purposes of this application, the terms "expression construct," "expression vector," and "vector," are used interchangeably to demonstrate the application of the invention in a
15 general, illustrative sense, and are not intended to limit the invention.

"Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, which may include a promoter operably linked to the nucleotide sequence of interest that may be operably linked to termination signals. The coding region usually codes for a functional
20 RNA of interest, for example an miRNA. The expression cassette including the nucleotide sequence of interest may be chimeric. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of a regulatable promoter that initiates
25 transcription only when the host cell is exposed to some particular stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development.

Such expression cassettes can include a transcriptional initiation region linked to a nucleotide sequence of interest. Such an expression cassette is provided with a plurality of
30 restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and

used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like. Reporter genes that encode for easily assayable proteins are also well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. For example, reporter genes include the chloramphenicol acetyl transferase gene (cat) from Tn9 of *E. coli* and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

“Functional RNA” refers to sense RNA, antisense RNA, ribozyme RNA, siRNA, or other RNA that may not be translated but yet has an effect on at least one cellular process.

“Regulatory sequences” are nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences that may be a combination of synthetic and natural sequences. As is noted herein, the term “suitable regulatory sequences” is not limited to promoters. However, some suitable regulatory sequences useful in the present invention will include, but are not limited to constitutive promoters, tissue-specific promoters, development-specific promoters, regulatable promoters and viral promoters.

“5' non-coding sequence” refers to a nucleotide sequence located 5' (upstream) to the coding sequence. It is present in the fully processed mRNA upstream of the initiation codon and may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

“3' non-coding sequence” refers to nucleotide sequences located 3' (downstream) to a coding sequence and may include polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The term “translation leader sequence” refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader

sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

“Promoter” refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which directs and/or controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription.

“Promoter” includes a minimal promoter that is a short DNA sequence comprised of a TATA- box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. “Promoter” also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors that control the effectiveness of transcription initiation in response to physiological or developmental conditions. Examples of promoters that may be used in the present invention include the mouse U6 RNA promoters, synthetic human H1RNA promoters, SV40, CMV, RSV, RNA polymerase II and RNA polymerase III promoters.

The “initiation site” is the position surrounding the first nucleotide that is part of the transcribed sequence, which is also defined as position +1. With respect to this site all other sequences of the gene and its controlling regions are numbered. Downstream sequences (i.e., further protein encoding sequences in the 3' direction) are denominated positive, while upstream sequences (mostly of the controlling regions in the 5' direction) are denominated negative.

Promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation are referred to as “minimal or core promoters.” In the presence of a suitable transcription factor, the minimal promoter

functions to permit transcription. A “minimal or core promoter” thus consists only of all basal elements needed for transcription initiation, e.g., a TATA box and/or an initiator.

“Constitutive expression” refers to expression using a constitutive or regulated promoter. “Conditional” and “regulated expression” refer to expression controlled by a regulated promoter.

“Operably-linked” refers to the association of nucleic acid sequences on single nucleic acid fragment so that the function of one of the sequences is affected by another. For example, a regulatory DNA sequence is said to be “operably linked to” or “associated with” a DNA sequence that codes for an RNA or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

“Expression” refers to the transcription and/or translation of an endogenous gene, heterologous gene or nucleic acid segment, or a transgene in cells. For example, in the case of antisense oligonucleotides or miRNAs, expression may refer to the transcription of the oligonucleotide or miRNA only. In addition, expression refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. Expression may also refer to the production of protein.

“Altered levels” refers to the level of expression in transgenic cells or organisms that differs from that of normal or untransformed cells or organisms.

“Overexpression” refers to the level of expression in transgenic cells or organisms that exceeds levels of expression in normal or untransformed cells or organisms.

“Antisense inhibition” refers to antisense RNA transcripts capable of suppressing the expression of miRNA or protein from an endogenous gene or a transgene.

“Transcription stop fragment” refers to nucleotide sequences that contain one or more regulatory signals, such as polyadenylation signal sequences, capable of terminating transcription. Examples include the 3' non-regulatory regions of genes encoding nopaline synthase and the small subunit of ribulose biphosphate carboxylase.

The terms “cis-acting sequence” and “cis-acting element” refer to DNA or RNA sequences whose functions require them to be on the same molecule. An example of a cis-acting sequence on the replicon is the viral replication origin.

The terms “trans-acting sequence” and “trans-acting element” refer to DNA or RNA sequences whose function does not require them to be on the same molecule.

“Chromosomally-integrated” refers to the integration of a foreign gene or nucleic acid construct into the host DNA by covalent bonds. Where genes are not “chromosomally integrated” they may be “transiently expressed.” Transient expression of a gene refers to the expression of a gene that is not integrated into the host chromosome but functions independently, either as part of an autonomously replicating plasmid or expression cassette, for example, or as part of another biological system such as a virus.

“Transformed,” “transduced,” “transgenic” and “recombinant” refer to a host cell into which a heterologous nucleic acid molecule has been introduced. As used herein the term “transfection” refers to the delivery of DNA into eukaryotic (e.g., mammalian) cells. The term “transformation” is used herein to refer to delivery of DNA into prokaryotic (e.g., E. coli) cells. The term “transduction” is used herein to refer to infecting cells with viral particles. The nucleic acid molecule can be stably integrated into the genome generally known in the art. Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially mismatched primers, and the like. For example, “transformed,” “transformant,” and “transgenic” cells have been through the transformation process and contain a foreign gene integrated into their chromosome. The term “untransformed” refers to normal cells that have not been through the transformation process.

A “host cell” is a cell that has been transformed/transfected, or is capable of transformation/transfection, by an exogenous nucleic acid molecule. Host cells containing the transformed/transfected nucleic acid fragments are referred to as “transgenic” cells.

“Genetically altered cells” denotes cells which have been modified by the introduction of recombinant or heterologous nucleic acids (e.g., one or more DNA constructs or their RNA counterparts) and further includes the progeny of such cells which retain part or all of such genetic modification.

As used herein, the term “derived” or “directed to” with respect to a nucleotide molecule means that the molecule has complementary sequence identity to a particular molecule of interest.

Recombinant DNA can be readily introduced into the host cells, e.g., mammalian, bacterial, yeast or insect cells via an expression vector by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a cell having the recombinant DNA stably integrated into its genome or existing as an episomal element, so that the DNA molecules, or sequences of the present invention are expressed by the host cell. Preferably, the DNA is introduced into host cells via a vector. The host cell is preferably of

eukaryotic origin, e.g., plant, mammalian, insect, yeast or fungal sources, but host cells of non-eukaryotic origin may also be employed.

Physical methods to introduce a preselected DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. For mammalian gene therapy, as described herein below, it is desirable to use an efficient means of inserting a copy gene into the host genome. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Patent Nos. 5,350,674 and 5,585,362. Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable viral expression vectors are available for transferring exogenous nucleic acid material into cells. The selection of an appropriate expression vector to express a therapeutic agent for a particular condition amenable to gene silencing therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation.

In another embodiment, an expression vector may be in the form of a plasmid, which is transferred into the target cells by one of a variety of methods: physical (e.g., microinjection, electroporation, scrape loading, microparticle bombardment) or by cellular uptake as a chemical complex (e.g., calcium or strontium co-precipitation, complexation with lipid, complexation with ligand). Several commercial products are available for cationic liposome complexation including Lipofectin™ (Gibco-BRL, Gaithersburg, Md.) and Transfectam™ (Promega®, Madison, Wis.). However, the efficiency of transfection by these methods is highly dependent on the nature of the target cell and accordingly, the conditions for optimal transfection of nucleic acids into cells using the herein-mentioned procedures must be optimized. Such optimization is within the scope of one of ordinary skill in the art without the need for undue experimentation.

In one embodiment, cells are transfected or transduced or otherwise genetically modified *in vivo*. The cells from the mammalian recipient are transduced or transfected *in vivo* with a vector containing exogenous nucleic acid material for expressing a heterologous (e.g., recombinant) gene encoding a therapeutic agent (e.g., a miR-9 and/or miR-25 inhibitor) and the therapeutic agent is delivered *in situ*, for example, injecting the vector into the recipient..

As used herein, “exogenous nucleic acid material” refers to a nucleic acid or an oligonucleotide, either natural or synthetic, which is not naturally found in the cells; or if it is naturally found in the cells, is modified from its original or native form. Thus, “exogenous nucleic acid material” includes, for example, a non-naturally occurring nucleic acid that can be transcribed into an anti-sense RNA, as well as a “heterologous gene” (i.e., a gene encoding a protein that is not expressed or is expressed at biologically insignificant levels in a naturally-occurring cell of the same type). To illustrate, a synthetic or natural gene encoding human erythropoietin (EPO) would be considered “exogenous nucleic acid material” with respect to human peritoneal mesothelial cells since the latter cells do not naturally express EPO. Still another example of “exogenous nucleic acid material” is the introduction of only part of a gene to create a recombinant gene, such as combining a regulatable promoter with an endogenous coding sequence via homologous recombination.

To confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; “biochemical” assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

To detect and quantitate RNA produced from introduced recombinant DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the recombinant DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the RNA or peptide products of the introduced recombinant DNA sequences or evaluating the phenotypic changes brought about by the expression of the introduced recombinant DNA segment in the host cell.

The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

The word "or" as used herein means any one member of a particular list and also
5 includes any combination of members of that list.

Ranges can be expressed herein as from "about" or "approximately" one particular value, and/or to "about" or "approximately" another particular value. When such a range is expressed, a further aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the
10 antecedent "about," or "approximately," it will be understood that the particular value forms a further aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint and independently of the other endpoint. It is also understood that there are a number of values disclosed herein and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For
15 example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that each unit between two particular units is also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

As used herein, the terms "optional" or "optionally" mean that the subsequently described event or circumstance may or may not occur and that the description includes
20 instances where said event or circumstance occurs and instances where it does not.

As used herein, the term "comprising" can include the aspects "consisting of" and "consisting essentially of."

As used herein, the term "sample" is meant a tissue or organ from a subject; a cell (either within a subject, taken directly from a subject, or a cell maintained in culture or from a
25 cultured cell line); a cell lysate (or lysate fraction) or cell extract; or a solution containing one or more molecules derived from a cell or cellular material (e.g. a polypeptide or nucleic acid). A sample may also be any body fluid or excretion (for example, but not limited to, blood, urine, stool, saliva, tears, bile) that contains cells or cell components.

As used herein, the term "subject" refers to the target of administration, e.g., a human.
30 Thus the subject of the disclosed methods can be a vertebrate, such as a mammal, a fish, a bird, a reptile, or an amphibian. The term "subject" also includes domesticated animals (e.g., cats, dogs, etc.), livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), and laboratory animals (e.g., mouse, rabbit, rat, guinea pig, fruit fly, etc.). In one aspect, a subject is a mammal. In another aspect, a subject is a human. The term does not denote a particular age or sex. Thus,

adult, child, adolescent and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

As used herein, the term "patient" refers to a subject afflicted with a disease or disorder. The term "patient" includes human and veterinary subjects. In some aspects of the disclosed methods, the "patient" has been diagnosed with a need for treatment for vascular disease, such as, for example, prior to the administering step.

"Inhibit," "inhibiting" and "inhibition" mean to diminish or decrease an activity, response, condition, disease, or other biological parameter. This can include, but is not limited to, the complete ablation of the activity, response, condition, or disease. This may also include, for example, a 10% inhibition or reduction in the activity, response, condition, or disease as compared to the native or control level. Thus, in an aspect, the inhibition or reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to native or control levels. In an aspect, the inhibition or reduction is 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90-100% as compared to native or control levels. In an aspect, the inhibition or reduction is 0-25, 25-50, 50-75, or 75-100% as compared to native or control levels.

"Modulate", "modulating" and "modulation" as used herein mean a change in activity or function or number. The change may be an increase or a decrease, an enhancement or an inhibition of the activity, function or number.

The terms "alter" or "modulate" can be used interchangeable herein referring, for example, to the expression of a nucleotide sequence in a cell means that the level of expression of the nucleotide sequence in a cell after applying a method as described herein is different from its expression in the cell before applying the method.

"Promote," "promotion," and "promoting" refer to an increase in an activity, response, condition, disease, or other biological parameter. This can include but is not limited to the initiation of the activity, response, condition, or disease. This may also include, for example, a 10% increase in the activity, response, condition, or disease as compared to the native or control level. Thus, in an aspect, the increase or promotion can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or more, or any amount of promotion in between compared to native or control levels. In an aspect, the increase or promotion is 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90-100% as compared to native or control levels. In an aspect, the increase or promotion is 0-25, 25-50, 50-75, or 75-100%, or more, such as 200, 300, 500, or 1000% more as compared to native or control levels. In an aspect, the increase or promotion can be greater than 100 percent as compared to native or control levels, such as

100, 150, 200, 250, 300, 350, 400, 450, 500% or more as compared to the native or control levels.

As used herein, the terms "disease" or "disorder" or "condition" are used interchangeably referring to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with a person. A disease or disorder or condition can also related to a distemper, ailing, ailment, malady, disorder, sickness, illness, complaint, affection.

The invention will now be illustrated by the following non-limiting Example.

EXAMPLE 1

Coordinated microRNA silencing of Nox4 NADPH oxidase and myocardin regulates smooth muscle cell dedifferentiation in vascular disease

The transcription factor myocardin plays a critical role in the regulation of smooth muscle cell (SMC) phenotypic switching in vascular disease (FIGS. 1 and 2). The Nox4 NADPH oxidase has been implicated in SMC differentiation and its expression associate with changes in myocardin levels (FIG. 3). However, the molecular basis of Nox4 regulation and the mechanisms by which vascular injury result in molecular reprogramming by myocardin are poorly understood. It was hypothesized that microRNAs (miR) mediate changes in Nox4 expression and regulate expression of genes implicated in SMC differentiation (FIG. 4). Analysis of the 3'UTR of Nox4 identified putative miR-9 and miR-25 binding sites, which were confirmed with luciferase reporter assays (FIG. 10). Treatment of human SMCs with a miR-9 or miR-25 mimic (1) silenced Nox4 mRNA and decreased ROS levels (FIGS. 5 and 6); (2) suppressed myocardin mRNA expression (FIG. 7) and serum responses factor; (3) decreased expression of multiple differentiation genes; and (4) was sufficient to induce cell migration. Expression of miR-9 and miR-25 was increased in cultured human SMCs and human pulmonary artery segments after treatment with TNF- α or thrombin, and in murine carotid artery ten days after partial carotid ligation. Interestingly, despite silencing by a miR-25 mimic, the myocardin 3'UTR binds miR-9 but not miR-25 (FIG. 9). It was found that miR-25 induced the expression of miR-9 and pretreatment with a miR-9 inhibitor prevented miR-25-mediated silencing of Nox4 and myocardin (FIG. 8). Suggesting a potential mechanism, miR-25 mimic caused demethylation of the miR-9 promoter (FIG. 11).

It was evaluated whether miR-9 and miR-25 contribute to SMC dedifferentiation in vascular disease (FIG. 12). The effect of growth factors and cytokines on miR-9 and miR-25

levels were investigated (FIG. 13). It was found that thrombin-mediated increase in miR-9 silences myocardin expression (FIG. 14). It was also found that miR-9 and miR-25 cause de-differentiation of SMCs (FIG. 15). It was observed that miR-9 and miR-25 levels are increased in SMCs derived from the neointimal (FIG. 16). It was also observed that miR-9 is increased in injured carotid artery and miR-9 inhibitor decreases intimal formation (FIG. 17).

Finally, a miR-9 inhibitor prevented the migration of cultured SMC to thrombin and decreased neointimal formation by more than 50% to carotid partial ligation in mice. It is concluded that following vascular injury (1) miR-25 induces epigenetic modifications resulting in the expression of miR-9; (2) miR-9 and miR-25 cooperate to silence Nox4; (3) Nox4 effects on SMC differentiation involve miR-9 regulation of myocardin; and (4) inhibition of miR-9 blocks neointimal formation (FIG. 18). These findings identify miR-9/Nox4 as part of a novel regulatory pathway and therapeutic target in vascular disease.

EXAMPLE 2

miR-9 and miR-25 Expression Levels Increased in Response to Carotid Injury

Mouse carotid arteries were subject to carotid injury and ten days later the levels of miR-9 and miR-25 were measured. Relative increases in expression levels of miR-9 and miR-25 were observed (FIG. 19). These data suggest that the subsequent increase in miR-9 and miR-25 acts to silence cellular pathways involved in SMC differentiation and quiescence, thereby allowing for SMC activation and development of vascular disease. This outcome is considered a normal response to vascular injury. These data further suggest that inhibiting miR-9 and/or-25 can prevent the development of vascular disease.

To test this hypothesis, mice were subject to carotid ligation with the application of miR-9 inhibitor or a control miRNA. One month later, arteries were sectioned and stained and the area of intimal growth measured (I/M = intimal area / medial area). Arteries treated with a miR-9 inhibitor at the time of injury demonstrated marked reduction in intimal growth (FIG. 20).

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors
5 intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly
10 contradicted by context.

CLAIMS

What is claimed is:

1. A method of treating vascular disease or tumor growth, or inhibiting neovascularization in a mammal, comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the mammal, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.
2. The method of claim 1, wherein the therapeutic agent increases or maintains Nox4 NADPH oxidase expression in a cell in the mammal, as compared to Nox4 NADPH oxidase expression in a cell in a mammal that was not administered the therapeutic agent.
3. The method of claim 1 or 2, wherein levels of ROS are increased or unchanged as compared to ROS levels in a cell in a mammal that was not administered the therapeutic agent.
4. The method of any one of claims 1-3, wherein myocardin mRNA expression is increased or unchanged as compared to myocardin mRNA expression in a cell in a mammal that was not administered the therapeutic agent.
5. The method of any one of claims 1-4, wherein cell migration is reduced or prevented as compared to cell migration in a cell in a mammal that was not administered the therapeutic agent.
6. The method of any one of claims 1-5, wherein the vascular disease is atherosclerosis, bypass graft stenosis or angioplasty/stent restenosis.
7. The method of any one of claims 1-5, wherein the tumor is a gastrointestinal, bladder, or uterine tumor.

8. The method of any one of claims 1-7, wherein the inhibitor reduces miR-9 or miR-25 expression and/or function.
9. The method of claim 8, wherein miR-9 or miR-25 expression and/or function is reduced by at least 10%.
10. The method of any one of claims 1-9, wherein the inhibitor is a miR-9 or miR-25 inhibitor.
11. The method of claim 10, wherein the miR-9 inhibitor is a miR-9 inhibitor comprising an oligonucleotide comprising 4 to 7 nucleotides that are complementary to miR-9 seed sequence 3'- UGGUUUC -'5 (SEQ ID NO: 9).
12. The method of claim 10, wherein the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to miR-9 seed sequence 3'- UGGUUUC -'5 (SEQ ID NO: 9).
13. The method of claim 10, wherein the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity with miR-9 UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO: 2).
14. The method of claim 10, wherein the miR-25 inhibitor is a miR-25 inhibitor comprising an oligonucleotide comprising 4 to 7 nucleotides that are complementary to miR-25 seed sequence 3'-CACGUUA-'5 (SEQ ID NO:7).
15. The method of claim 10, wherein the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to miR-25 seed sequence 3'-CACGUUA-'5 (SEQ ID NO:7).

16. The method of claim 10, wherein the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity with miR-25 CAUUGCACUUGUCUCGGUCUGA (SEQ ID NO: 8).
17. The method of any one of claims 11-16, wherein the oligonucleotide is between about 18 to about 25 nucleotides in length.
18. The method of any one of claims 11-16, wherein the oligonucleotide is chemically modified.
19. The method of any one of claims 11-16, wherein the oligonucleotide contains at least one modified nucleotide analog.
20. The method of claim 19, wherein the modified nucleotide analog is a 2' modified nucleotide.
21. The method of claim 19, wherein the modified nucleotide analog is a backbone-modified nucleotide.
22. The method of any one of claims 18 to 21, wherein oligonucleotide contains at least one locked nucleic acid.
23. The method of any one of claims 1-22, wherein the administration is by systemic or local release.
24. The method of claim 23, wherein the delivery is by intravenous delivery.
25. A method of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-9, comprising contacting the cell in vivo or in vitro with a miR-9 inhibitor.

26. A method of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-25, comprising contacting the cell in vivo or in vitro with a miR-25 inhibitor.
27. An article of manufacture comprising a solid substrate coated with therapeutic agent comprising a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.
28. The article of claim 27, wherein the solid substrate is a medical device.
29. The article of claim 28, wherein the medical device is a drug eluting arterial stent, a catheter, a catheter hub, a catheter port, or a non-degradable implant.
30. A method of treating vascular disease in a subject, the method comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.
31. A method of reducing or inhibiting tumor growth in a subject, the method comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.
32. A method of inhibiting neovascularization in a subject, the method comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the subject, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.
33. A method of preventing or reducing the risk of graft disease in a subject, the method comprising administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.

34. A method of preventing or reducing the risk of restenosis in a subject, the method comprising administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.
35. A method of preventing or reducing the risk of arterial restenosis or accelerated venous bypass disease in a subject, the method comprising administering to the subject an effective amount of a therapeutic agent, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and micro-RNA-25 (miR-25) inhibitor.
36. The method of claim 35, wherein the therapeutic agent is administered to the subject prior to, during or after vascular balloon angioplasty, deployment of an arterial stent, artero-venous fistula creation for hemodialysis, exposure to pulmonary toxins, hyperoxia/hypoxia involved in the development of pulmonary hypertension or organ transplant vasculopathy.
37. The method of any one of claims 30-35, wherein the therapeutic agent increases or maintains Nox4 NADPH oxidase expression in a cell in the subject compared to Nox4 NADPH oxidase expression in a cell in a subject that was not administered the therapeutic agent.
38. The method of any one of claims 30-35 and 37, wherein levels of ROS are increased or unchanged as compared to ROS levels in a cell in a subject that was not administered the therapeutic agent.
39. The method of any one of claims 30-35 or 37-38, wherein myocardin mRNA expression is increased or unchanged compared to myocardin mRNA expression in a cell in a subject that was not administered the therapeutic agent.
40. The method of any one of claims 30-35 or 37-39, wherein cell migration is reduced or prevented compared to cell migration in a cell in a subject that was not administered the therapeutic agent.

41. The method of any one of claims 30-35 or 37-40, wherein levels of serum response factor are increased or unchanged compared to levels of serum response factor in a cell in a subject that was not administered the therapeutic agent.
42. The method of any one of claims 30 or 37-41, wherein the vascular disease is atherosclerosis, bypass graft stenosis, angioplasty or stent restenosis.
43. The method of any one of claims 31 or 37-41, wherein the tumor is a gastrointestinal, bladder, or uterine tumor.
44. The method of any one of claims 30-41, wherein the therapeutic agent reduces miR-9 and/or miR-25 expression and/or function.
45. The method of claim 44, wherein miR-9 or/and miR-25 expression and/or function is reduced by at least 10%.
46. The method of any of claims 30-35, wherein the miR-9 inhibitor comprises an oligonucleotide comprising 4 to 7 nucleotides that are complementary to (SEQ ID NO: 9).
47. The method of any of claims 30-35, wherein the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to (SEQ ID NO: 9).
48. The method of any of claims 30-35, wherein the miR-9 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to (SEQ ID NO: 2).
49. The method of any of claims 30-35, wherein the miR-25 inhibitor comprises an oligonucleotide comprising 4 to 7 nucleotides that are complementary to (SEQ ID NO: 7).
50. The method of any of claims 30-35, wherein the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to (SEQ ID NO: 7).

51. The method of any of claims 30-35, wherein the miR-25 inhibitor comprises an oligonucleotide comprising a sequence having at least about 70% complementarity to (SEQ ID NO: 8).
52. The method of any one of claims 46-51, wherein the oligonucleotide is between about 18 to about 25 nucleotides in length.
53. The method of any one of claims 46-51, wherein the oligonucleotide is chemically modified.
54. The method of any one of claims 46-51, wherein the oligonucleotide contains at least one modified nucleotide analog.
55. The method of claim 54, wherein the modified nucleotide analog is a 2' modified nucleotide.
56. The method of claim 54, wherein the modified nucleotide analog is a backbone-modified nucleotide.
57. The method of any one of claims 53 to 56, wherein oligonucleotide contains at least one locked nucleic acid.
58. The method of any one of claims 30-57, wherein the administration is systemic or local.
59. The method of claim 30-57, wherein the administration is intravenous.
60. A method of improving contractility of a vascular smooth muscle cell that expresses miR-9 or miR-25, the method comprising contacting the cell in vivo or in vitro with a miR-9 inhibitor and miR-25 inhibitor of any of claims 46-51.
61. An article of manufacture comprising a solid substrate coated with a therapeutic agent comprising a micro-RNA-9 (miR-9) inhibitor and a micro-RNA-25 (miR-25) inhibitor.
62. The article of claim 61, wherein the solid substrate is a medical device.
63. The article of claim 62, wherein the medical device is a drug eluting arterial stent, a catheter, a catheter hub, a catheter port, or a non-degradable implant.

64. A method of decreasing dysfunction in a vascular smooth muscle cell that expresses miR-9 or miR-25, comprising contacting the cell in vivo or in vitro with a miR-9 inhibitor and a miR-25 inhibitor.
65. The method of any one of claims 1-64, wherein the micro-RNA-9 (miR-9) inhibitor and the micro-RNA-25 (miR-25) inhibitor are nucleic acid based.
66. The method of claims 1-65, wherein the micro-RNA-9 (miR-9) inhibitor and the micro-RNA-25 (miR-25) inhibitor are functional nucleic acids. (see document 2095462, paragraphs 207-221)
67. The method of claim 1-66, wherein the micro-RNA-9 (miR-9) inhibitor and the micro-RNA-25 (miR-25) inhibitor is an antisense molecule, aptamer, ribozyme, triplex forming molecules, or external guide sequence.

Figure 1

Development of Atherosclerosis

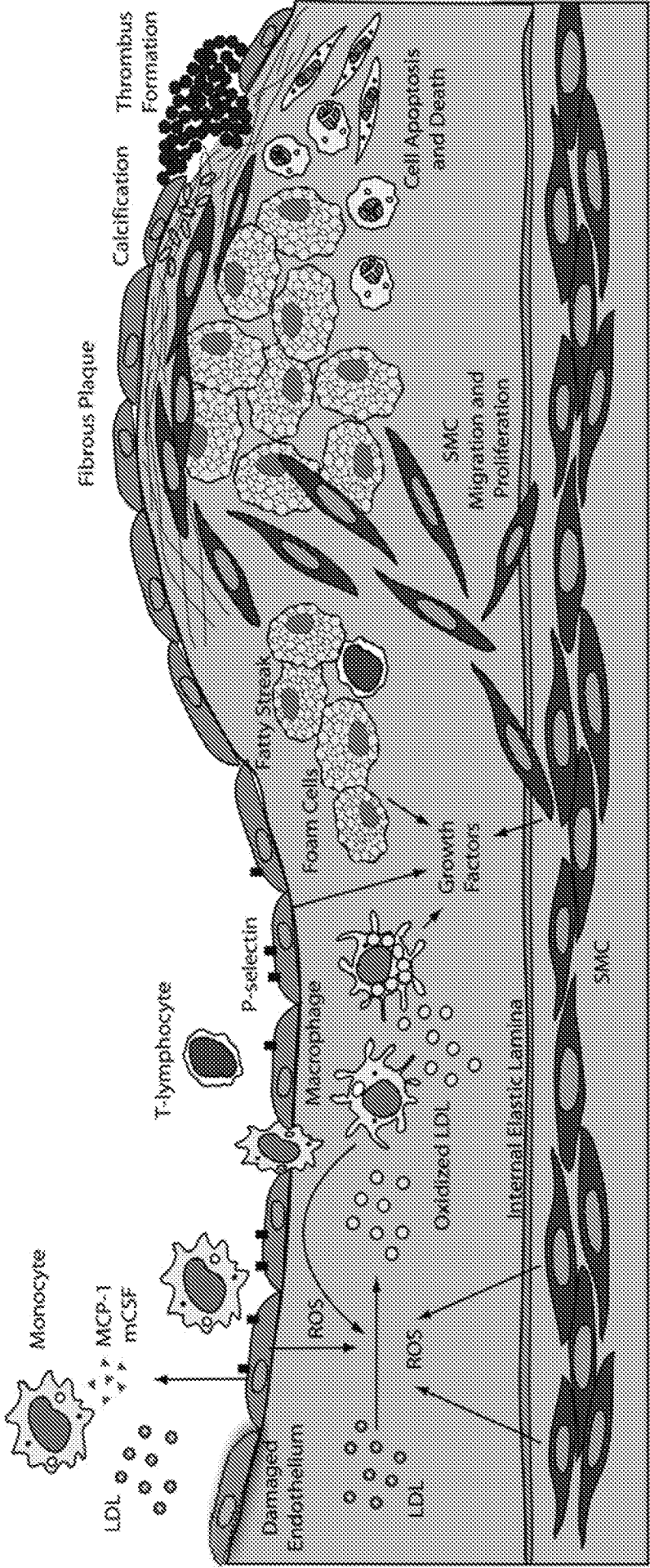


Figure 2

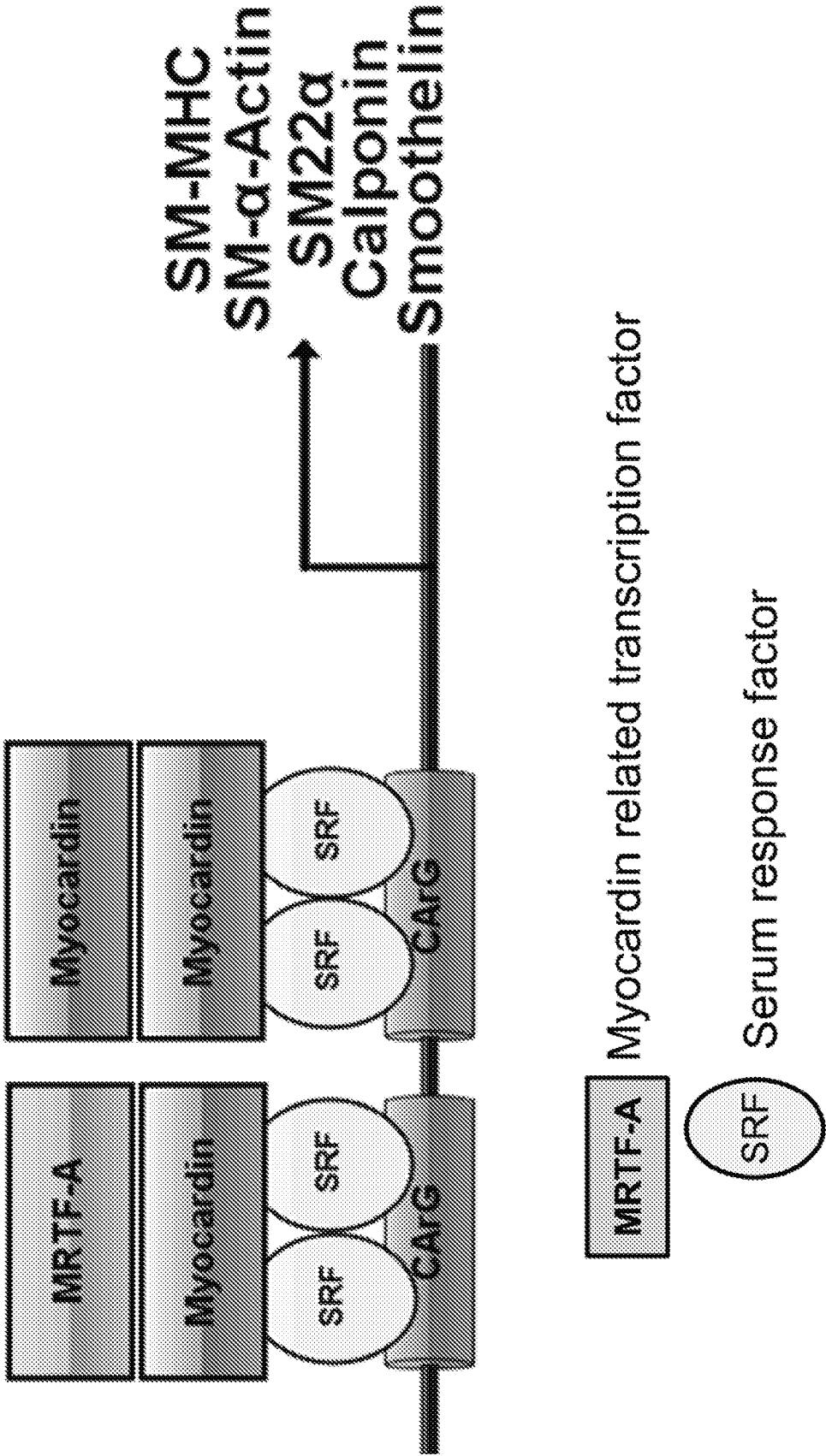


Figure 3

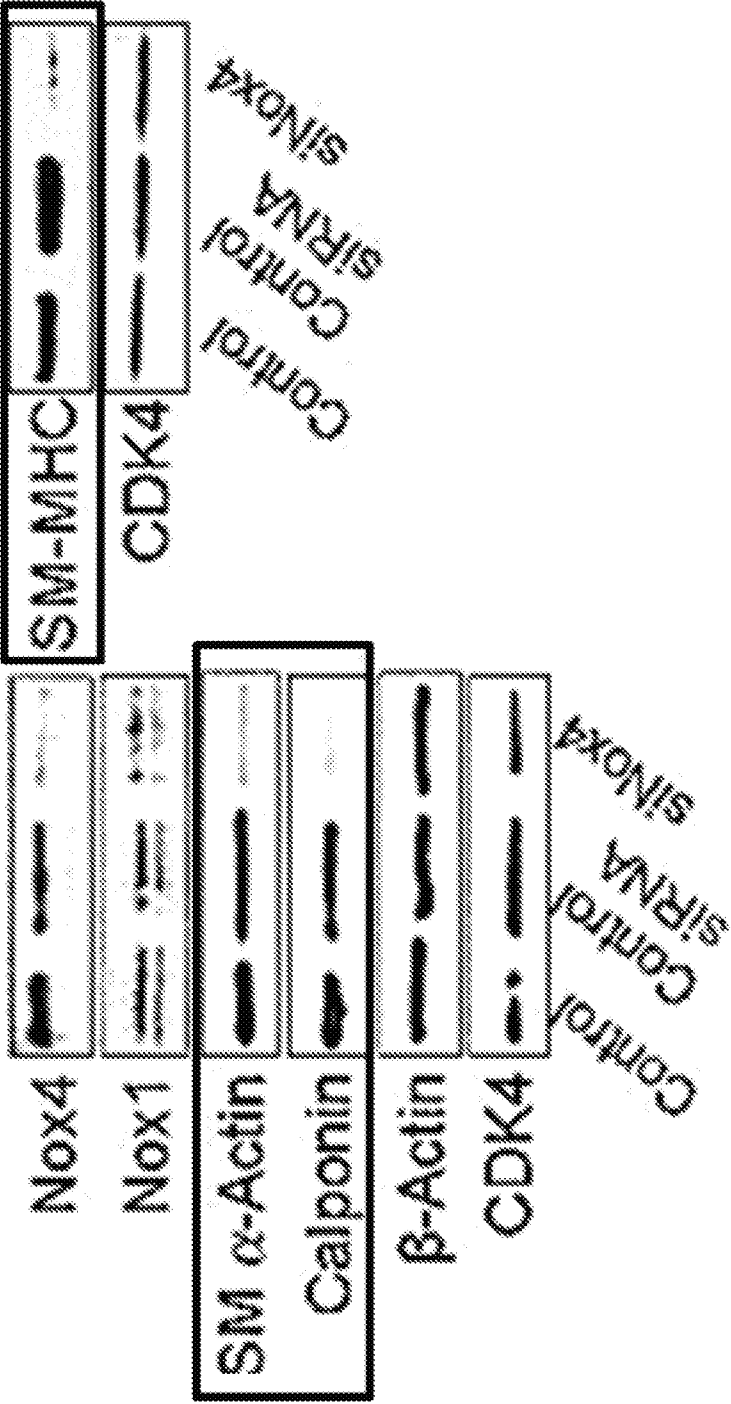


Figure 4

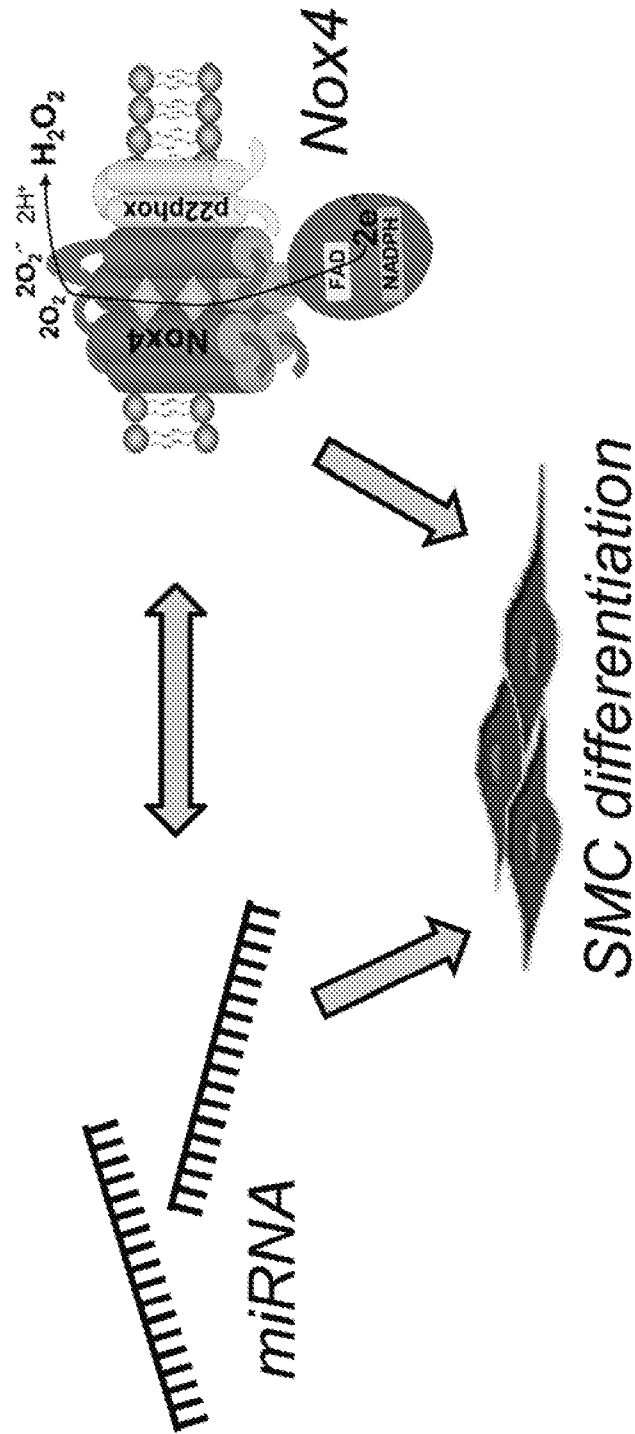
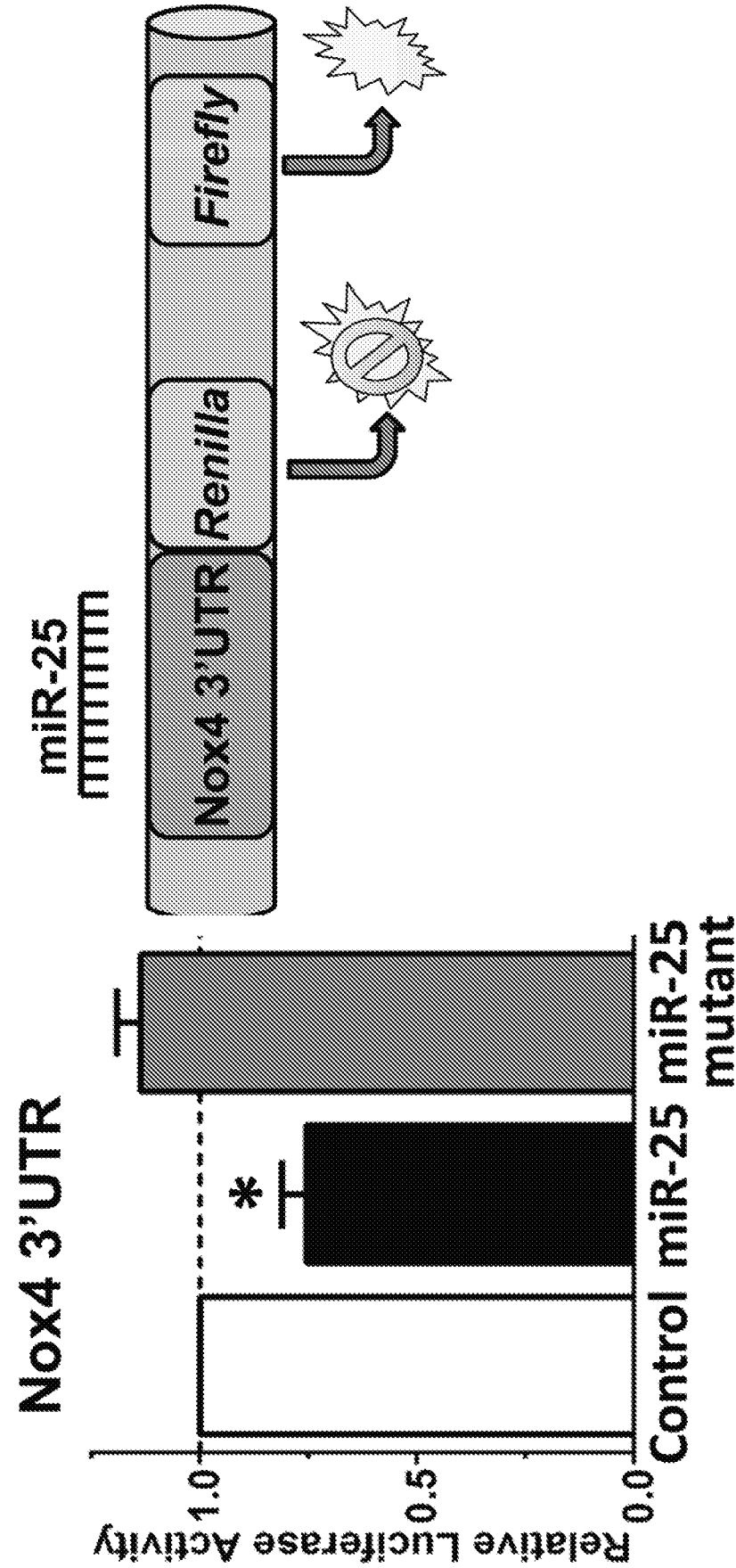
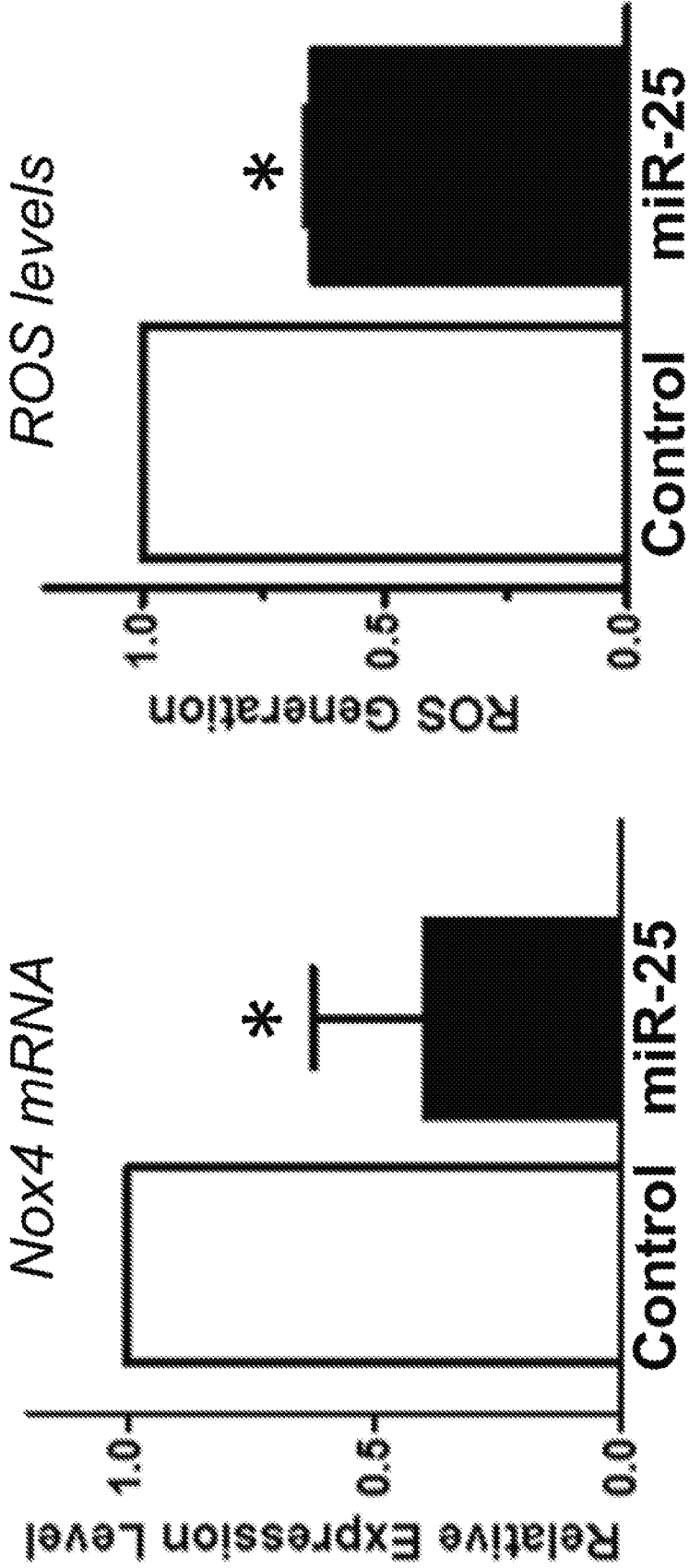
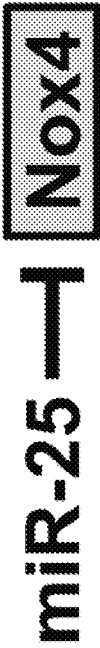


Figure 5



Human SMCs

Figure 6



Human SMCs

Figure 7

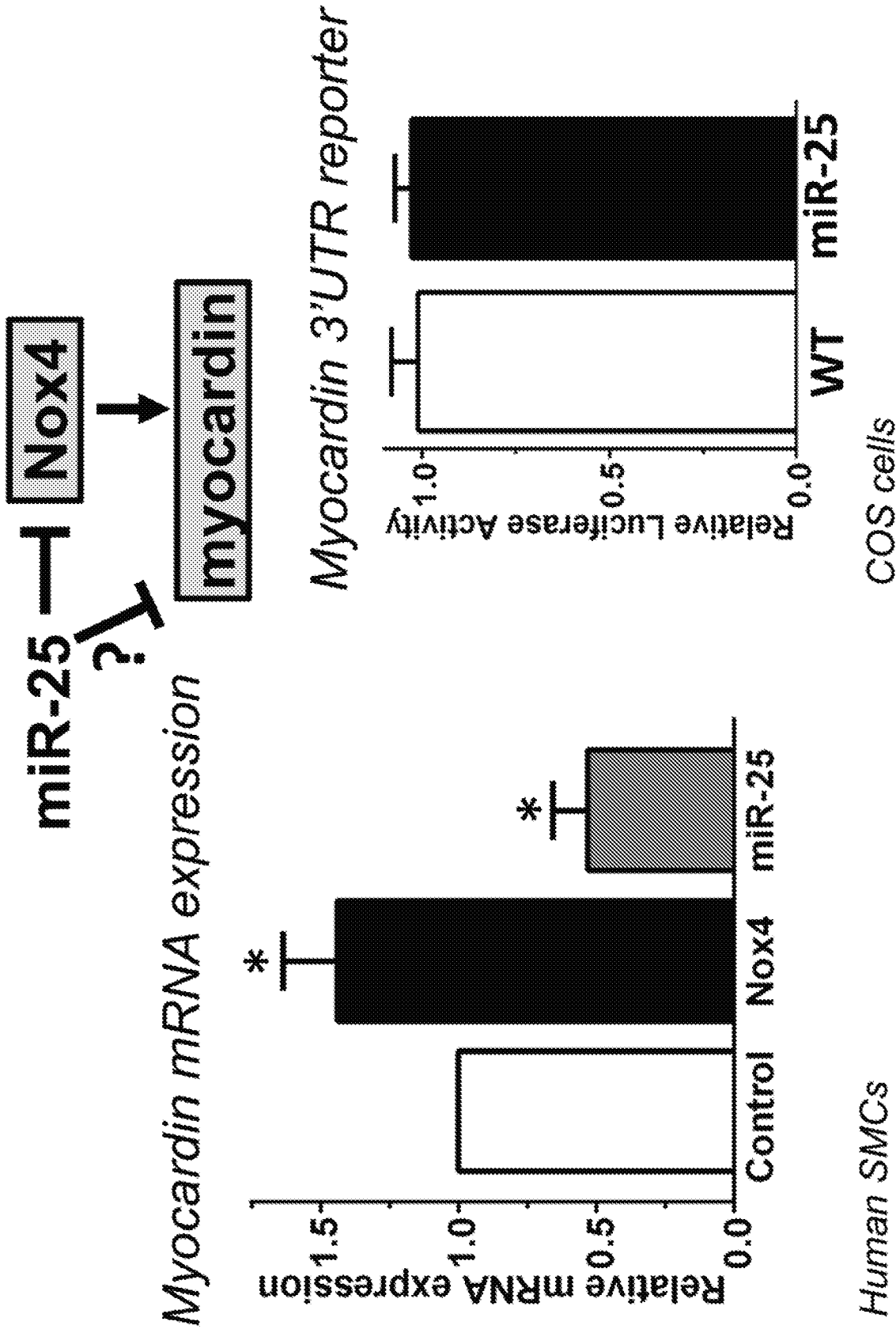


Figure 8

In response to miR-25

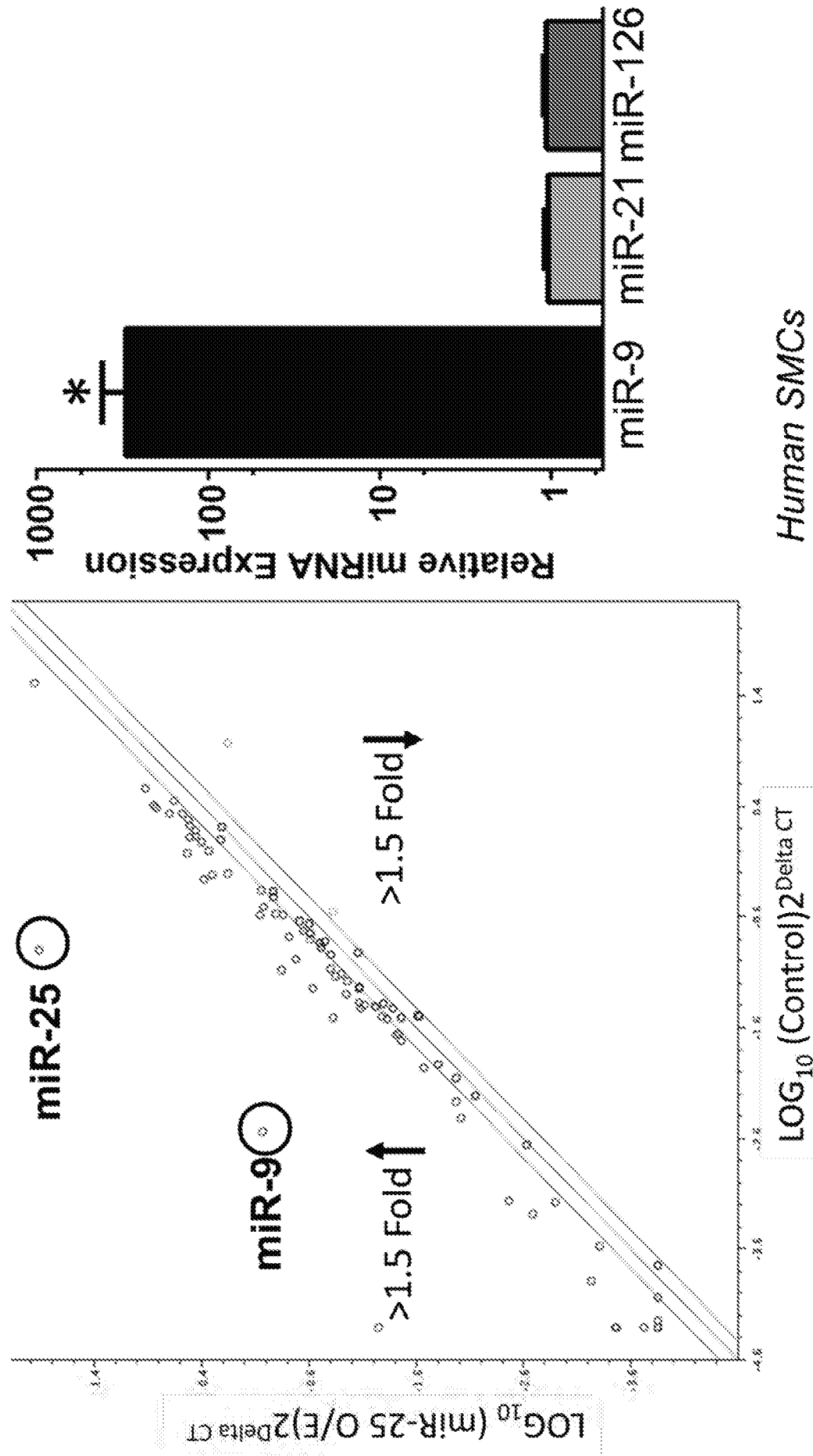


Figure 9

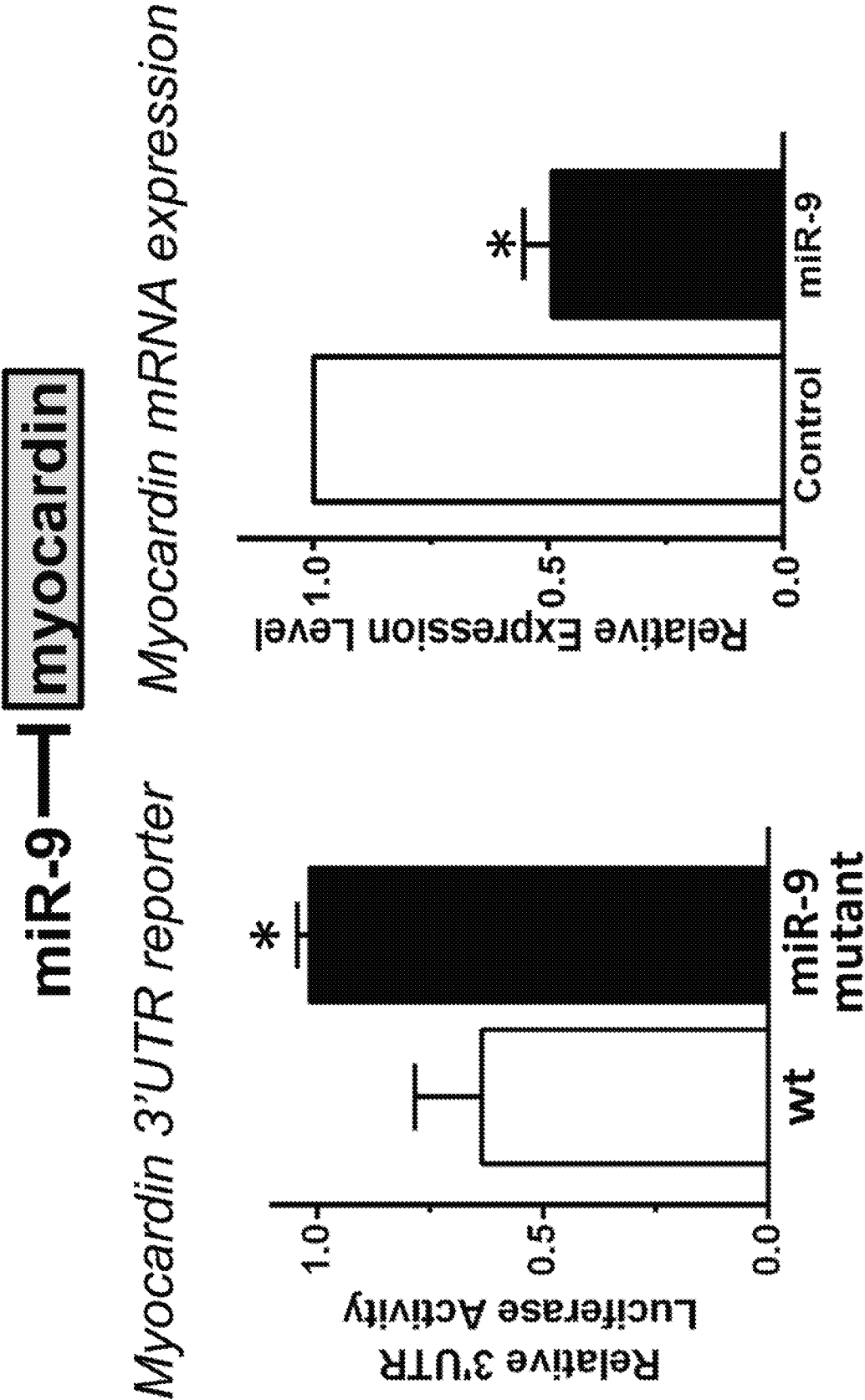


Figure 10

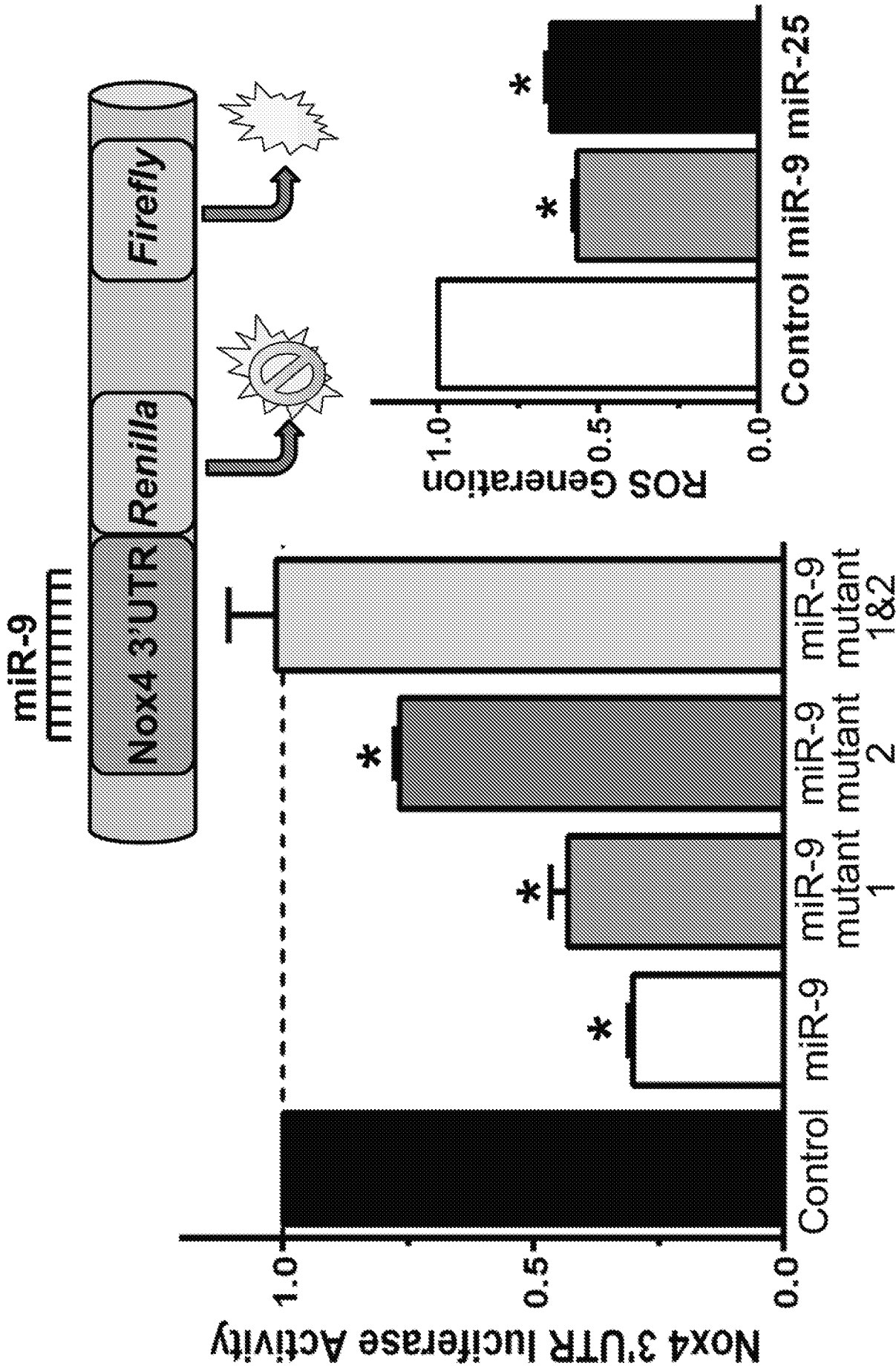


Figure 11

Bisulfite conversion and methylation-specific PCR

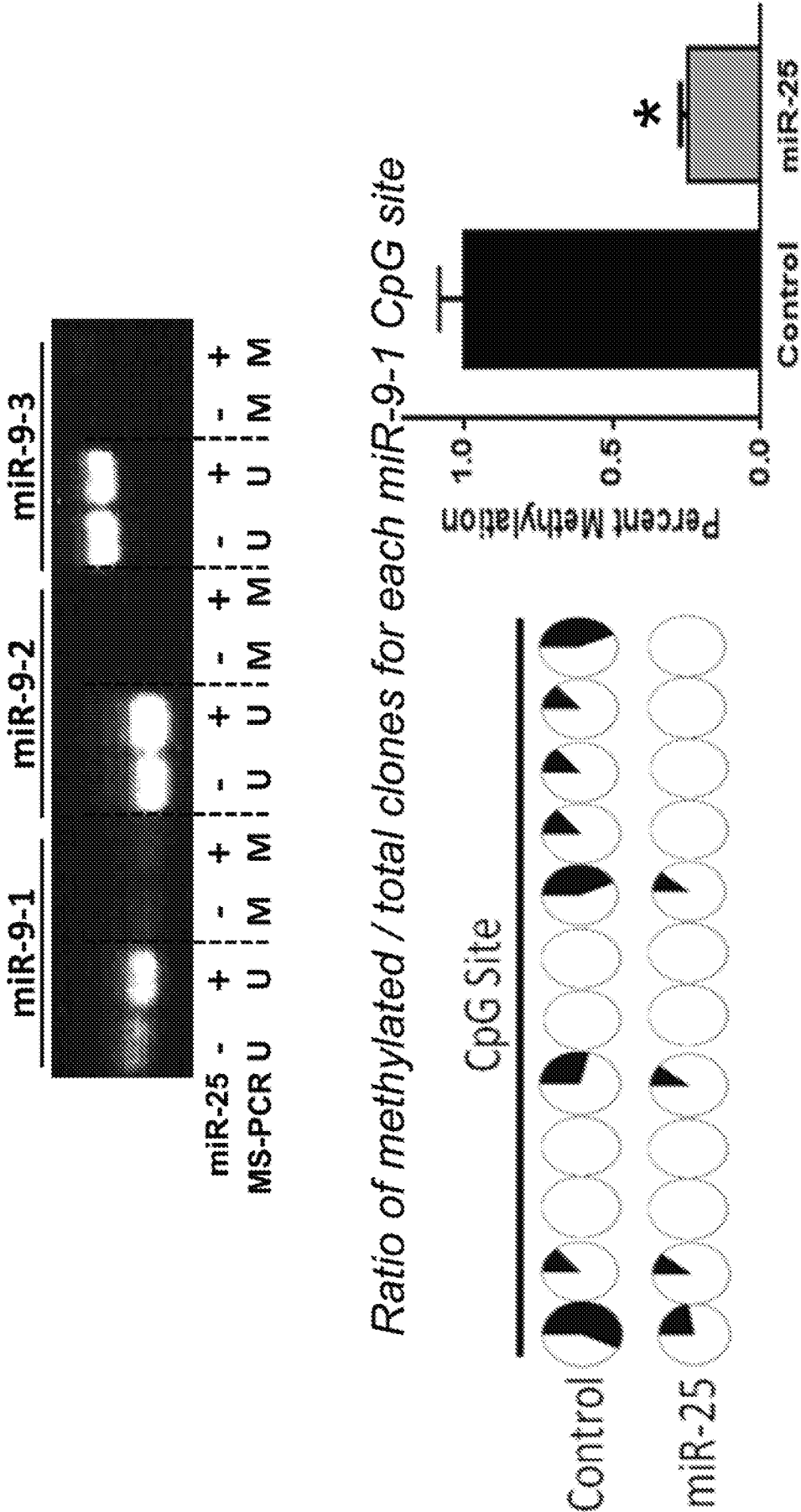


Figure 12

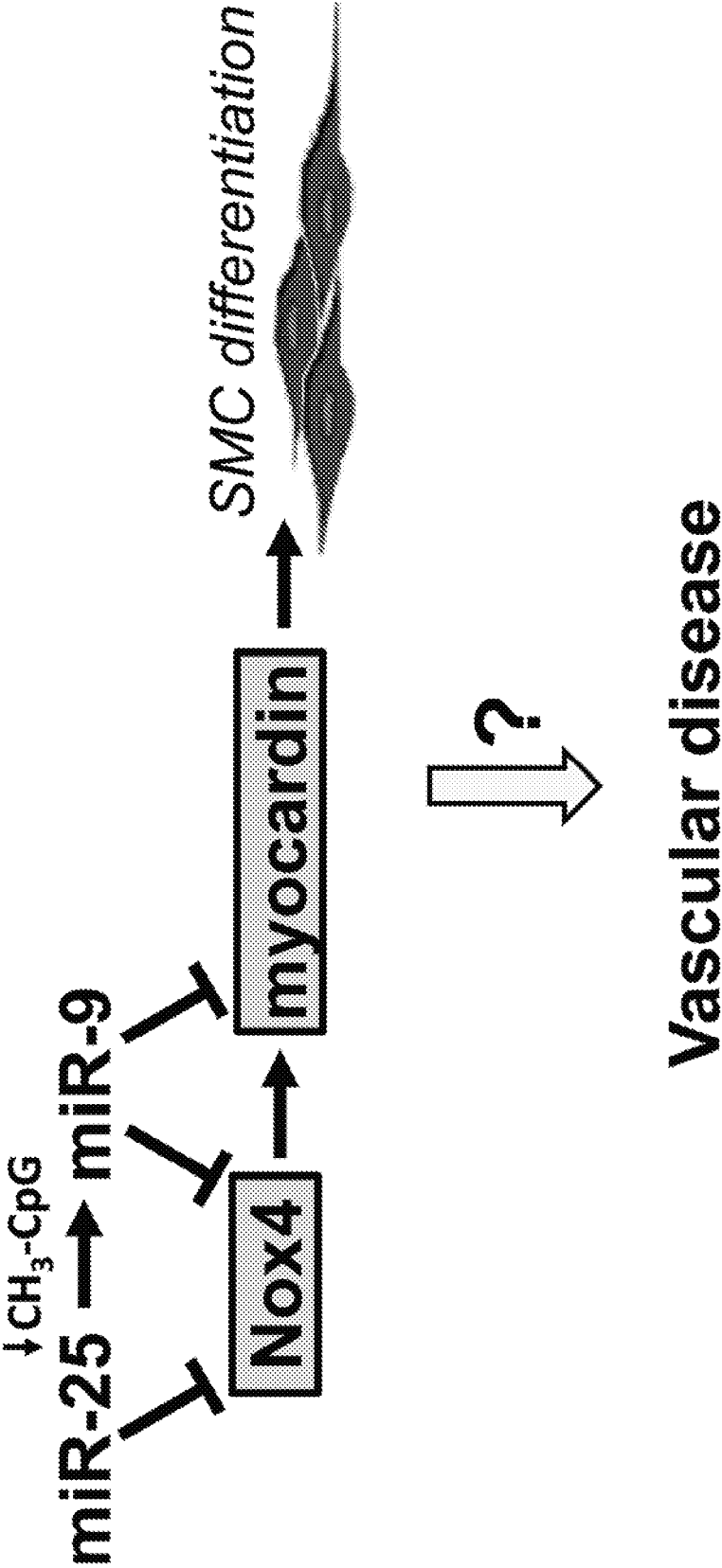
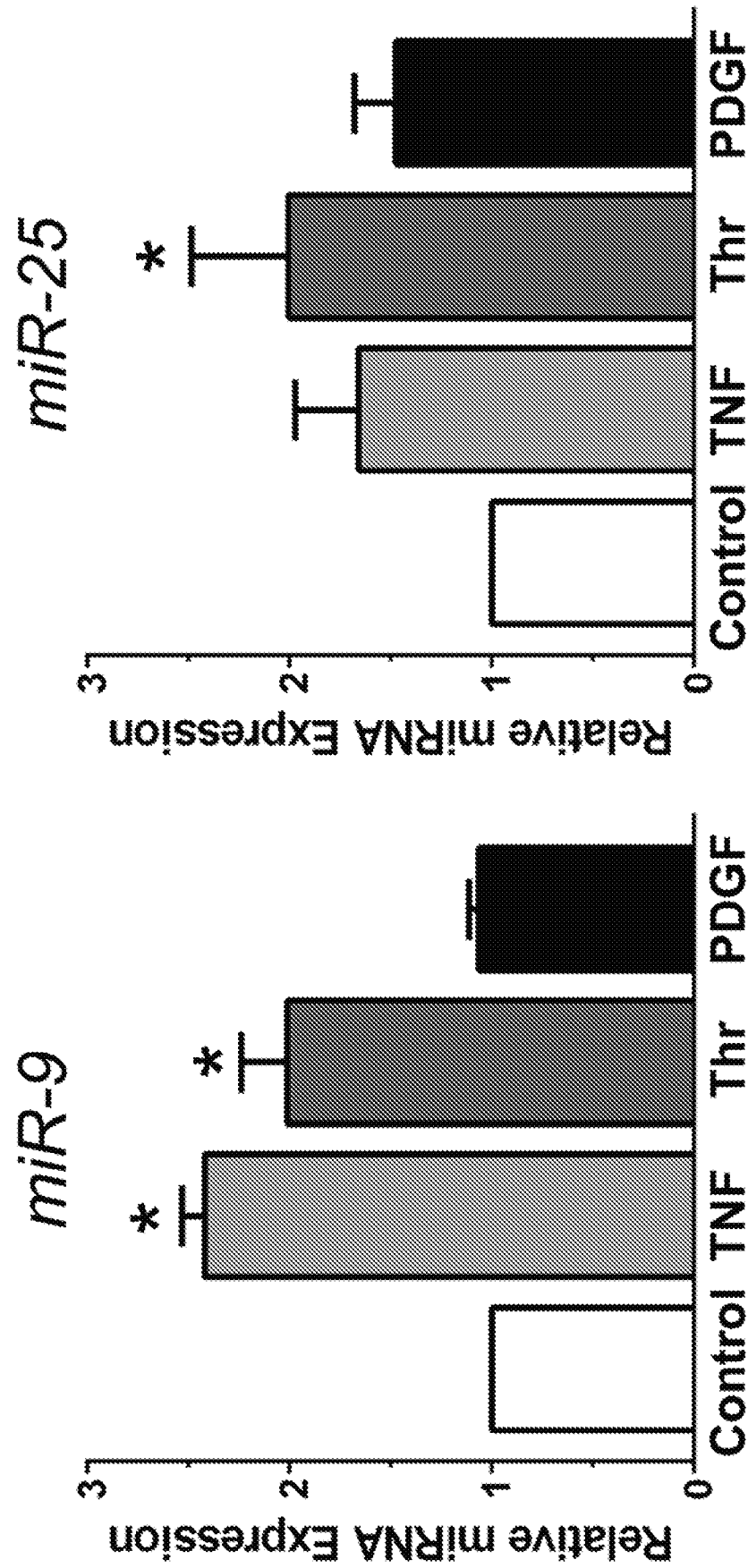
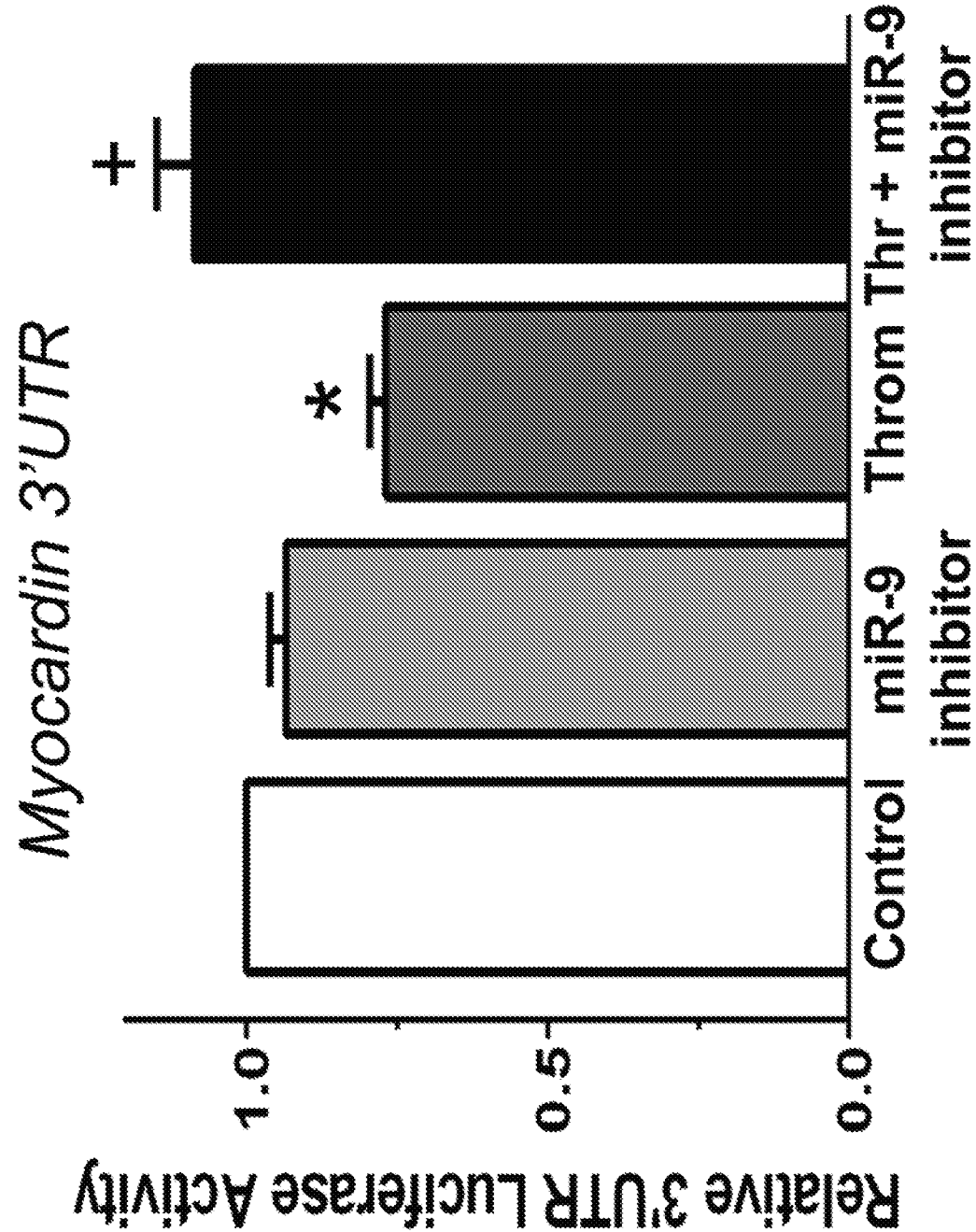


Figure 13



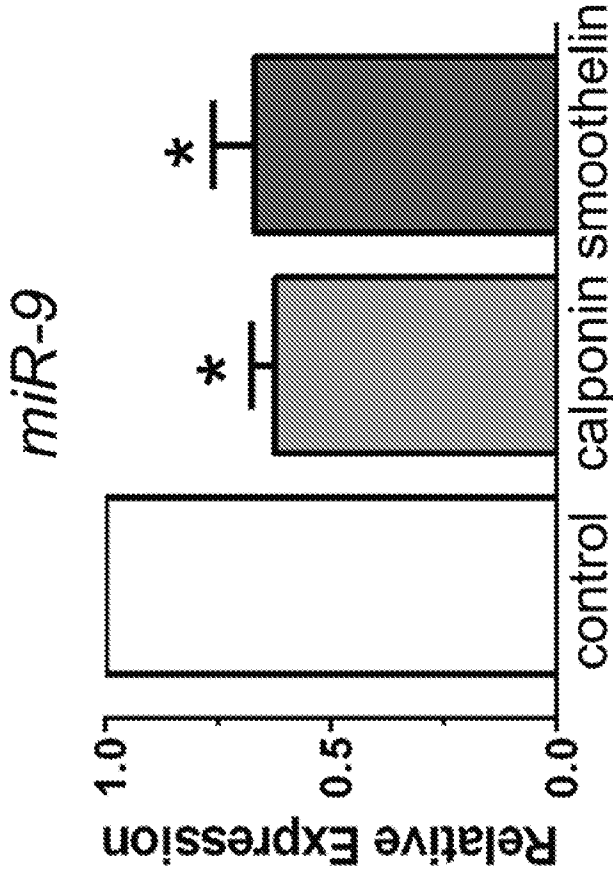
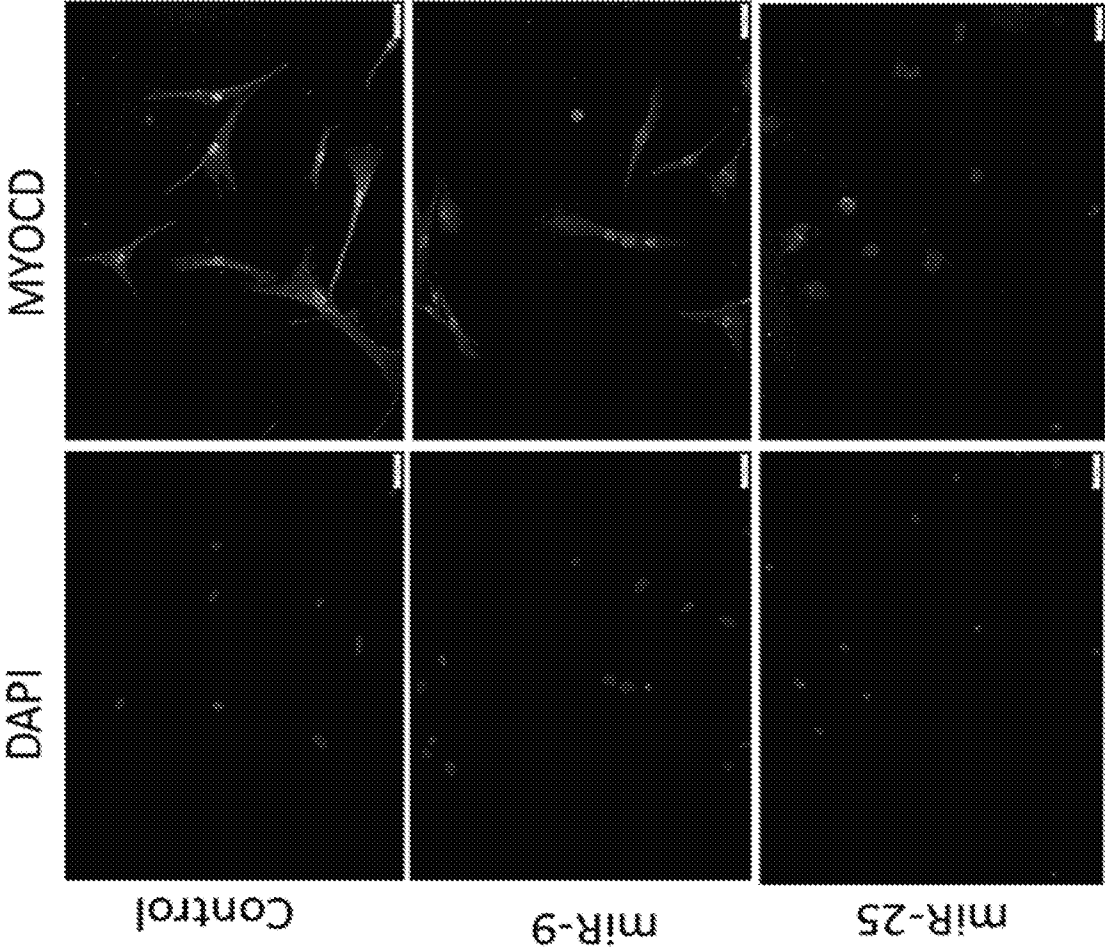
Human VSMC

Figure 14



HEK293

Figure 15



Human VSMC

Figure 16

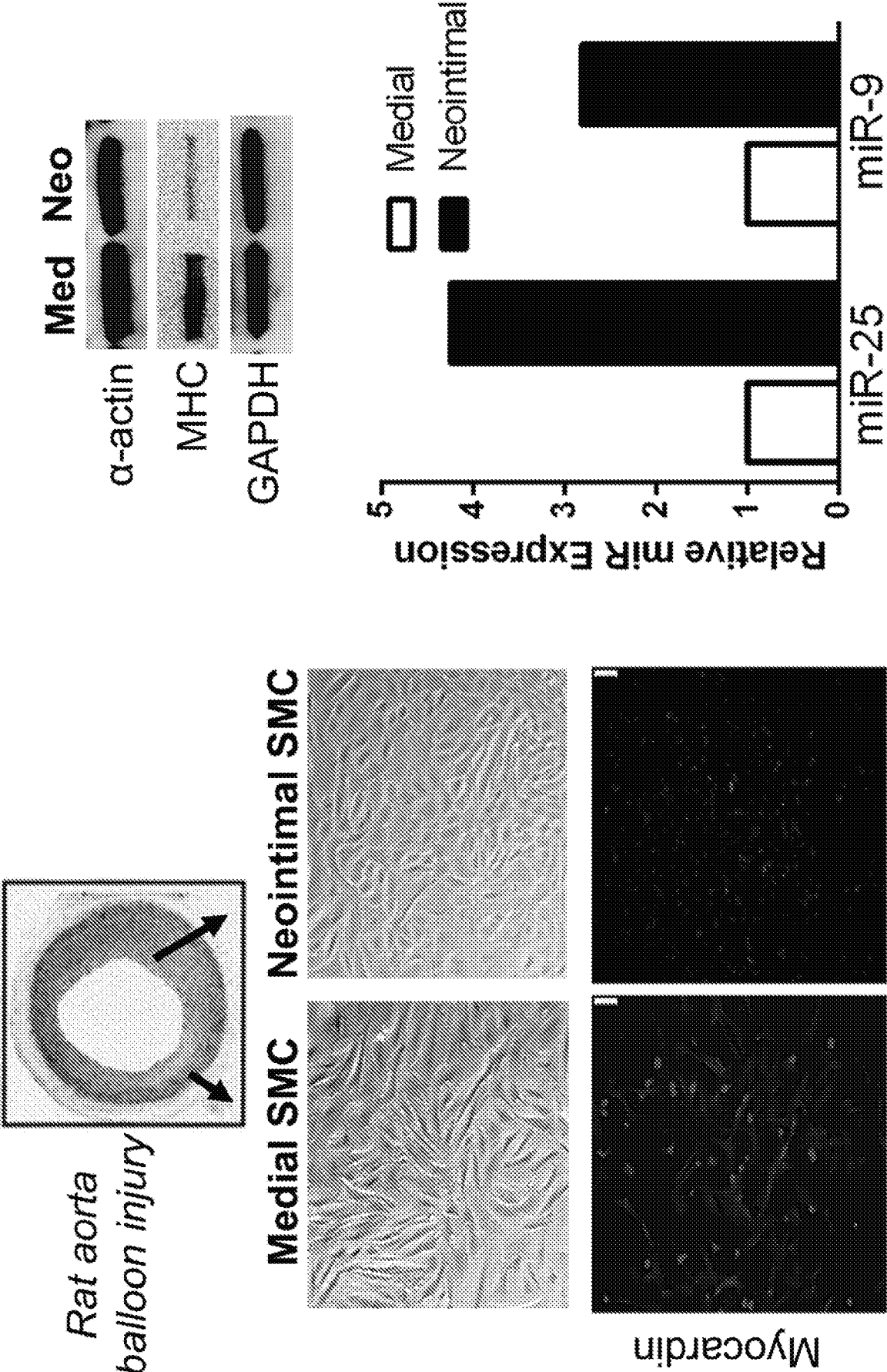
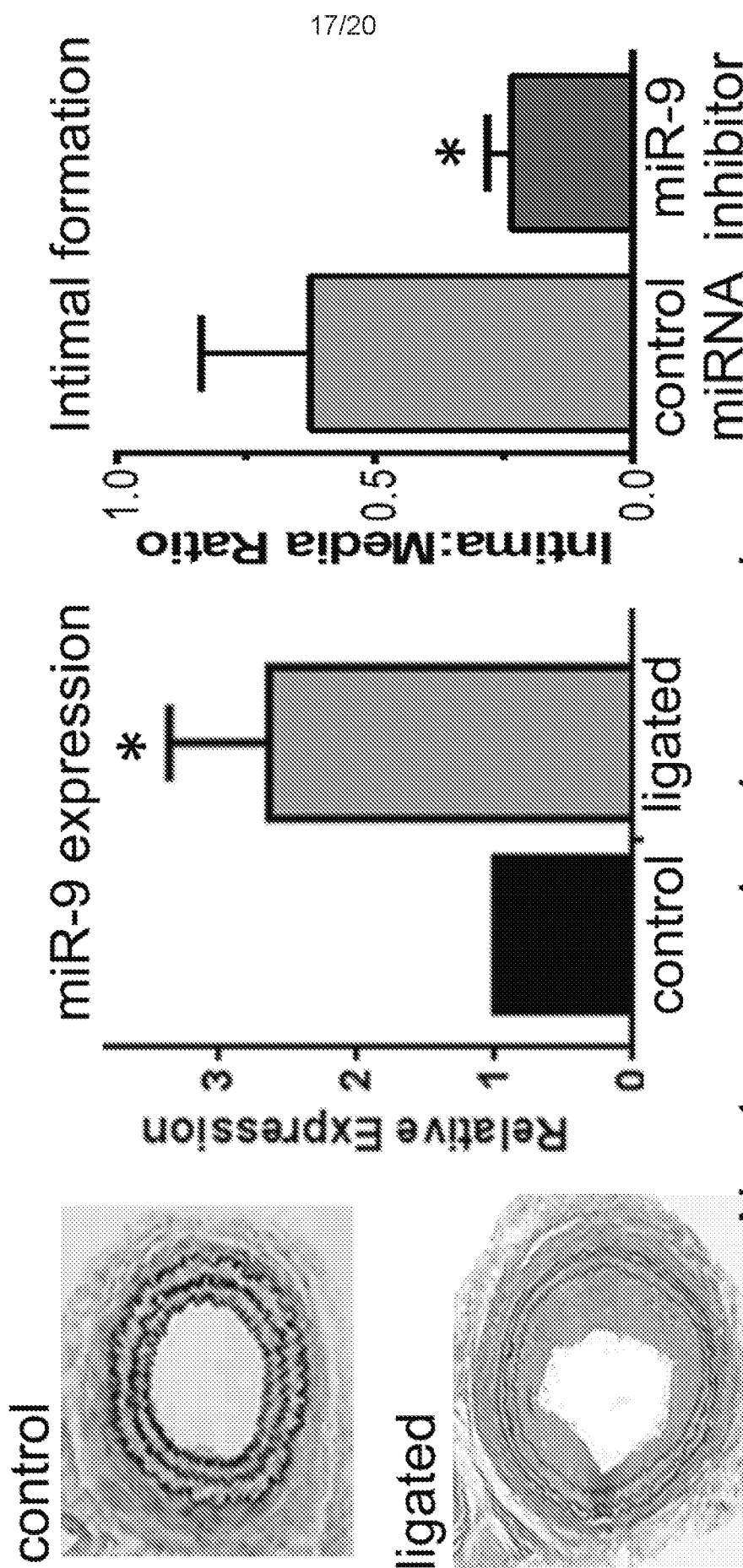


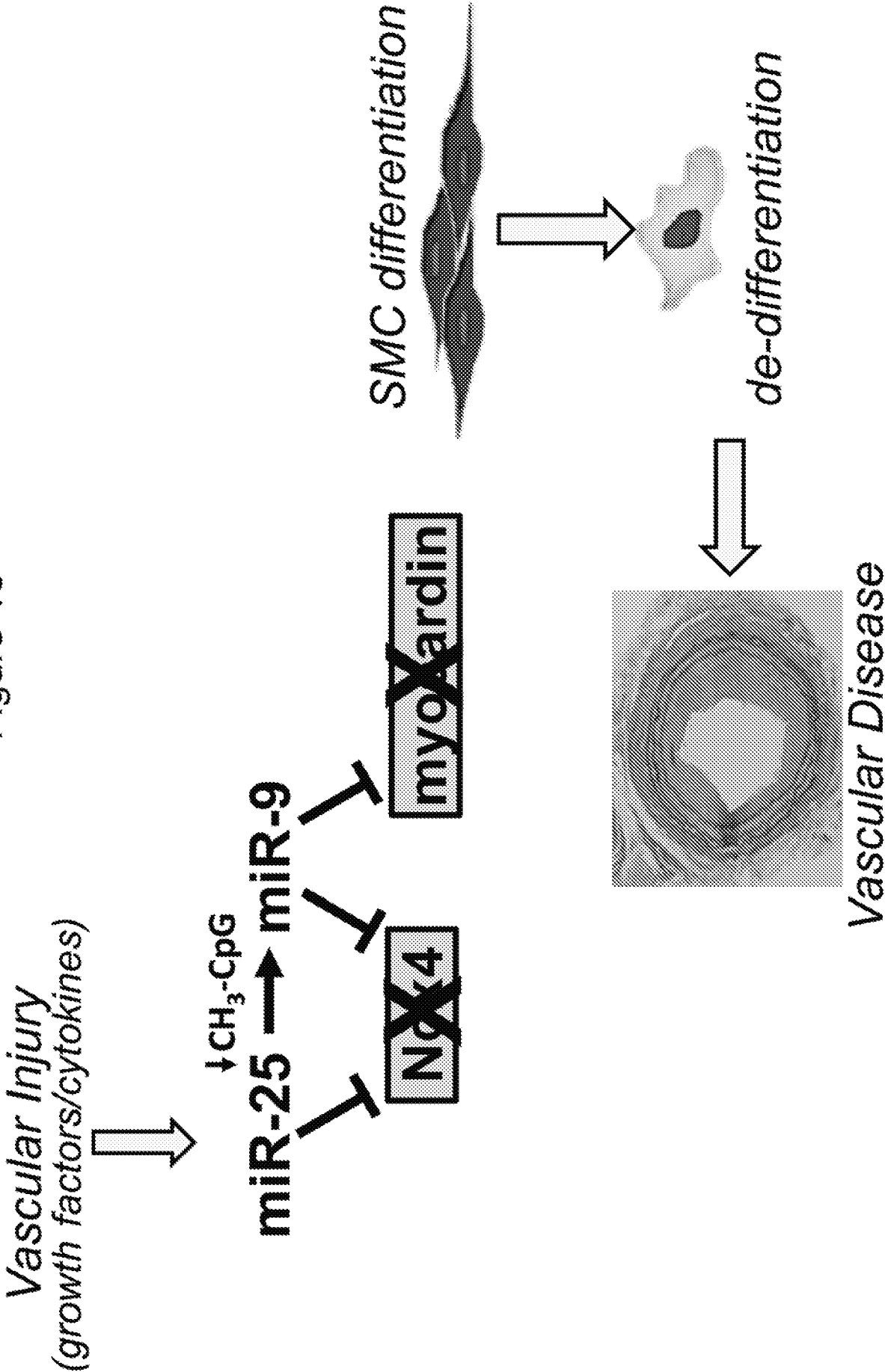
Figure 17

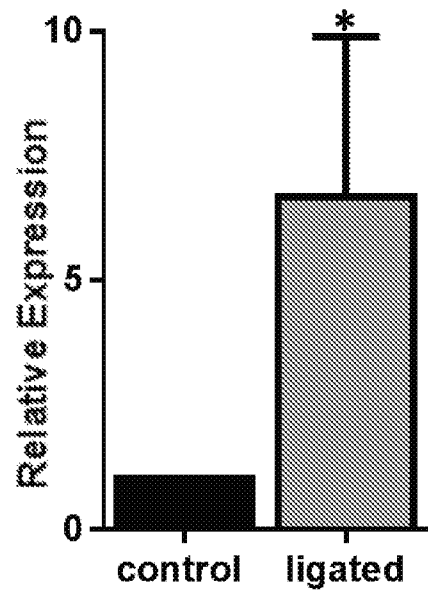
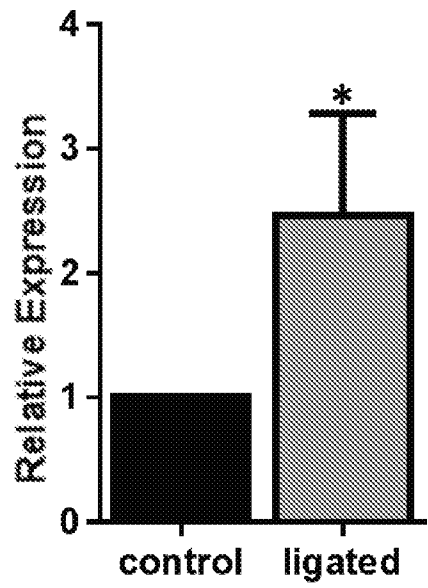
Mouse carotid artery

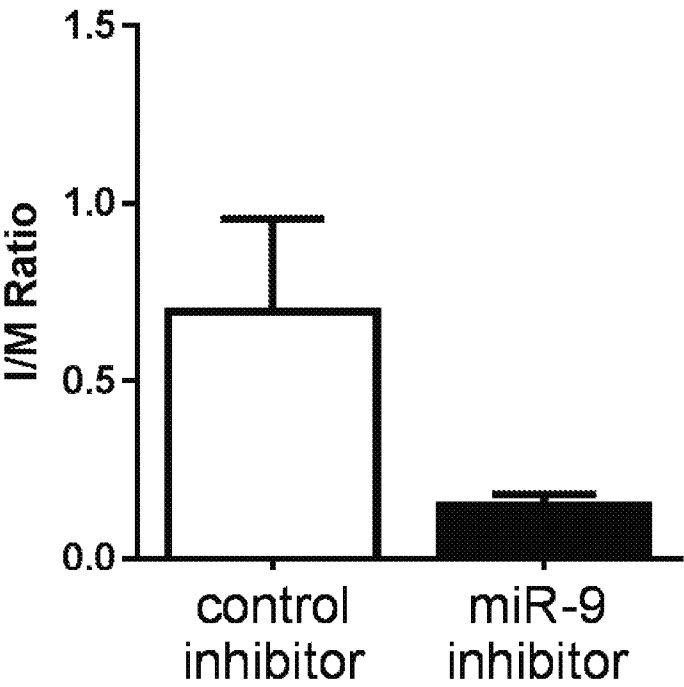
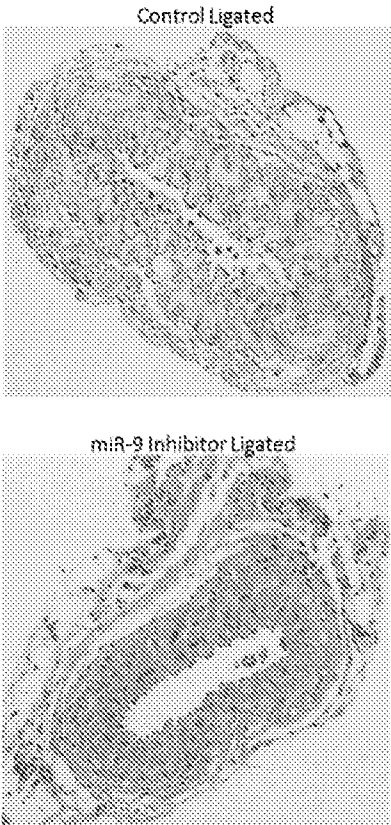


Nox4 expression decreased
(Schurmann, Eur Heart J, 2015)

Figure 18







INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/62821

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/113; A61K 31/7088 (2017.01)

CPC - C12N 2310/141, 2310/113, 15/11, 15/1136, 2320/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU et al. Requirement of miR-9-dependent regulation of Myocd in PSMCs phenotypic modulation and proliferation induced by hepatopulmonary syndrome rat serum. J Cell Mol Med October 2015 Vol 19 No 10 Pages 2453-2461. Especially abstract, "; pg 2454 col 2 para 3, "; Pg 2456 col 2 para 3	1, 25
Y		2, 3
Y	Fierro-Fernandez et al. miR-9-5p suppresses pro-fibrogenic transformation of fibroblasts and prevents organ fibrosis by targeting NOX4 and TGFBR2. EMBO Rep Epub 27 August 2015 Vol 16 No 10 Pages 1358-1377. Especially abstract Pg 1359 col 2 para 2.	2, 3

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 January 2017

Date of mailing of the international search report

17 APR 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/62821

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/62821

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-24, 38-45, 52-60, 65-67
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
---go to Extra Sheet for continuation---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-3, 25 limited to miR-9 inhibitor

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

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-----continuation of Box III (Lack of Unity of Invention)-----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-3, 25, 26, 30-37, 46-51, 64, drawn to a method of treating vascular disease or tumor growth or inhibiting neovascularization in a mammal with the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.

The method will be searched to the extent to encompass the first named therapeutic agent, miR-9 inhibitor. It is believed that claims 1-3, 25, 64 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass miR-9 inhibitor. Additional therapeutic agent(s) will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected therapeutic agent(s). Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: miR-25 inhibitor (claims 1-3, 25, 26, 30-37, 46-51, 64). [NOTE: claim 64 is excluded from the first invention because the first invention is limited to miR-9 inhibitor ONLY]

Group II: Claims 27-29, 61-63, drawn to a composition comprising a solid substrate coated with a miR-9 and/or miR-25 inhibitor.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of administering a miR-9 and/or miR-25 inhibitor(s) therapeutic agent or administering a vector comprising an expression cassette operably linked to a promoter and encoding miR-9 and/or miR-25, not required by Group II.

Group II has the special technical feature of a composition comprising a solid substrate coated with a miR-9 and/or miR-25 inhibitor, not required by Group I+.

Among the inventions listed as Groups I+ are the specific miRNA sequences recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among the sequences.

Common Technical Features:

1. Group I+ claims share the common technical feature of a method of treating vascular disease or tumor growth, or inhibiting neovascularization in a mammal, comprising administering an effective amount of a therapeutic agent or a vector comprising an expression cassette comprising a promoter operably linked to a nucleic acid encoding a therapeutic agent to the mammal, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor and/or micro-RNA-25 (miR-25) inhibitor.

2. Groups I+ and II share the common technical feature of a miR-9 and/or miR-25 inhibitor(s).

However, said common technical feature does not represent a contribution over the prior art, and is obvious over the publication titled "Requirement of miR-9-dependent regulation of Myocd in PSMCs phenotypic modulation and proliferation induced by hepatopulmonary syndrome rat serum" by XU et al. (hereinafter "Xu") [published October 2015 in J Cell Mol Med Vol 19 No 10 Pages 2453-2461], in view of US 9,051,571 B2 to Asuragen (hereinafter "Asuragen") [published 9 June 2015].

As to common technical features #1 and #2, Xu teaches a method of treating vascular disease or tumor growth, or inhibiting neovascularization in a mammal, comprising administering an effective amount of a therapeutic agent to the mammal, wherein the therapeutic agent comprises a micro-RNA-9 (miR-9) inhibitor. (Abstract: "Hepatopulmonary syndrome (HPS) is characterized by a triad of severe liver disease, intrapulmonary vascular dilation and hypoxaemia. Pulmonary vascular remodeling (PVR) is a key feature of HPS pathology. Our previous studies have established the role of the pulmonary artery smooth muscle cell (PASMC) phenotypic modulation and proliferation in HPS-associated PVR. Myocardin, a robust transcriptional coactivator of serum response factor, plays a critical role in the vascular smooth muscle cell phenotypic switch.....Furthermore, miRNA functional analysis and luciferase reporter assay demonstrated that miR-9 effectively regulated myocardin expression by directly binding to its 3'-untranslated region. Both the KNOCKDOWN of miR-9 and overexpression of myocardin effectively attenuated the HPS rat serum-induced phenotype switch and proliferation of PSMCs"; Pg 2456 col 2 para 3; "To investigate whether miR-9 functions as a direct regulator of the HPS rat serum-induced PASMC phenotypic switch, miR-9 inhibitor was transfected into cultured PSMCs, and then treated with HPS rat serum for 48 hrs. As demonstrated by Western blot analysis, transfection with miR-9 INHIBITOR significantly repressed HPS rat serum-induced down-regulation of myocardin and phenotype markers SM-alpha-actin and SM-MHC (Fig.3A-C). These findings indicate that miR-9 functions as a potent regulator in HPS rat serum-induced PASMC phenotypic modulation"; pg 2454 col 2 para 3; "Ectopic expression of miR-9 in cultured PSMCs was achieved by transfection with miR-9 mimics or inhibitors (Qiagen)").

Furthermore, as to common technical features #1 and #2, Asuragen teaches a miR-25 inhibitor to block cell proliferation (col 11 ln 21-45; "In some embodiments, there is a method for reducing or inhibiting cell proliferation in a cell comprising introducing into or providing to the cell an effective amount of i) an miRNA inhibitor molecule.....any of the following may be included in the group from which the miRNA inhibitor molecule (i) may be chosen:.....miR-25"; col 12 ln 29-33; "Methods for reducing or inhibiting cell proliferation can be used as a treatment for diseases and conditions that include, but are not limited to, hyperproliferative diseases, such as cancer").

-----continued on next sheet-----

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-----continued from previous sheet-----

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.