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(54) Title: METHODS FOR MODULATING ADIPOCYTE EXPRESSION USING MICRORNA COMPOSITIONS

(57) Abstract: Disclosed are compositions and methods useful for modulating adipocyte differentiation and/or development. Also disclosed are compounds, compositions, and methods useful for altering the expression and/or activity of one or more genes involved in pathways relating to adipogenesis and/or adipocyte activity both in vitro and in vivo. In particular, the present invention provides small interfering nucleic acid molecules, and in particular, microRNA and miR-155-derived molecules, and methods for their use in altering the expression, regulation, or accumulation of one or more adipogenic or anti-adipogenic genes or a gene products in a mammal,

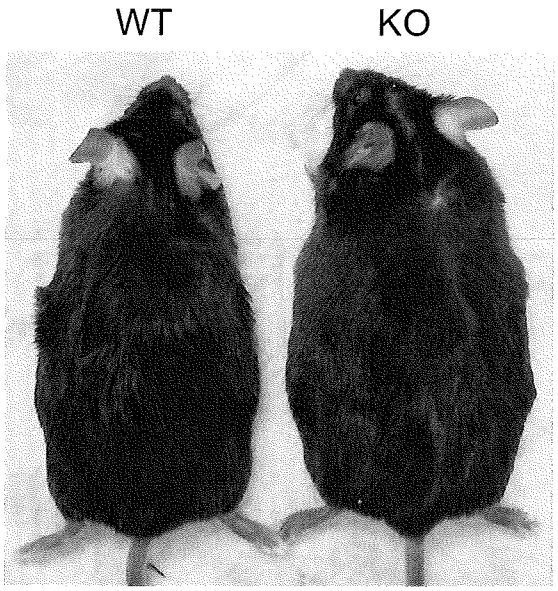

FIG. 27A

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DESCRIPTION

METHODS FOR MODULATING ADIPOCYTE EXPRESSION USING MICRORNA COMPOSITIONS

1. BACKGROUND OF THE INVENTION

1.1 CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to U.S. Provisional Patent Appl. No. 61/180,667, filed May 22, 2009, the entire contents of which is specifically incorporated herein in its entirety by express reference thereto.

1.2 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not Applicable.

1.3 NAMES OF THE PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not Applicable.

1.4 FIELD OF THE INVENTION

[0004] The present invention relates generally to the fields of medicine and molecular genetics. More particularly, it concerns compositions and methods for modulating adipocyte differentiation in mammalian cells. The invention also provides compounds and formulations for systemic and tissue-specific localization of molecules that affect one or more steps in a mammalian adipogenic pathway within particular cells or tissues of an animal. Such methods provide new treatment modalities for adipogenic conditions, including, without limitation, overeating, obesity, hyperlipidemia, excessive body weight gain, and abnormal fatty tissue formation and/or accumulation within the body of such animal.

1.5 DESCRIPTION OF RELATED ART

[0005] An estimated 54 percent of adults in the United States are overweight, according to the National Institutes of Health. Treating obesity and its related conditions, including high blood pressure, heart disease and diabetes, is estimated to cost more than $45 billion annually, according to an August 1996 article in *Scientific American*. With the incidence of obesity on the rise in the United States, there is a critical need to develop more effective therapies to reduce the health risks and alleviate the symptoms associated with obesity, overeating, excessive bodyweight gain, or excessive accumulation of fat.

[0006] Most current approaches used to achieve weight loss or control excessive body weight gain in an animal attempt to reduce caloric excess by a combination of caloric restriction (diet) and increased caloric demand (exercise). Caloric restriction by itself induces loss of both adipose and lean muscle mass. Conversely, excess caloric intake can result in lipid storage in several non-adipose tissues, usually with deleterious effects on the metabolic functions of these tissues.

[0007] New drugs recently introduced have been designed to augment standard weight loss approaches, and primarily function directly or indirectly as appetite suppressants to improve adherence to caloric restriction regimens. Serious side effects, however, have been associated with the use of all these drugs. PPARγ agonists, such as rosiglitazone and pioglitazone, are currently the only drugs routinely prescribed to the overweight and obese patient population that have direct effects on adipose tissue metabolism. Both drugs promote adipose redistribution from visceral to subcutaneous adipose tissue depots, but also have undesirable side effects, including generalized weight gain and/or edema.

[0008] Regulation of adipogenesis at the cellular level, however, for example, by inducing or repressing differentiation of preadipocytes to adipocytes, would provide a significantly enhanced method for controlling biochemical processes, such as, for example, the extent and/or tissue location for lipid deposition during excess caloric intake. What is desirable, therefore, are compositions and methods for their delivery to a selected mammal in need thereof using one or more systemic, localized, or cell-targeted delivery modalities to deliver agents capable of inducing, altering, controlling, modulating, or repressing at least one of adipocyte expression, adipocyte activity, and adipocyte differentiation.

2. BRIEF SUMMARY OF THE INVENTION

[0009] The present invention provides new and useful compositions, as well as methods of employing them that may advantageously modulate, alter, or ameliorate the expression of one or more gene(s), protein(s), or polypeptide(s) involved in adipogenesis in cells, tissues, and organs of interest within the body of an animal in need thereof using methodologies that involve the therapeutic delivery of one or more micro-RNA (miRNA) compositions (and in particular, micro RNA 155 [miR-155] compositions), to an animal in need thereof.

[0010] The invention advantageously capitalizes on the discovery by the present inventors that miR-155 plays an important and demonstrable role in at least one step of adipogenesis, adipocyte regulation and/or expression, and/or fat accumulation and distribution with the body of an animal. To exploit the therapeutic consequences of these observations, the

inventors have developed compositions that modulate, alter, or regulate one or more steps in the metabolic pathways involved in adipogenesis in an animal, and particularly in mammals such as humans. Preferably, the compositions include one or more of the following: (a) miR-155 compounds or analogs thereof; (b) compounds that regulate, control, increase, or induce endogenous miR-155 expression; and (c) synthetic miR-155 compounds or mimetics, inducers, or inhibitors thereof. From the scientific observations described herein, the inventors have developed miR-155-based treatment modalities that offer significant advantages over conventional therapies directed at controlling or eliminating excessive adipocyte differentiation, expression, or fat accumulation within the animal.

[0011] The use of natural or synthetic miR-155 compounds, miR-155 analogs, mimetics, or antisense compositions complementary thereto, permits the specificity of the miR-155 effect to be refined by altering the miR mimetic sequence(s) to selectively match one or more specific miR-155 targets while reducing the recognition and subsequent attenuation of other miR target genes whose attenuation could have less desired or deleterious effects. Since target gene recognition is based on sequence complementarity, this approach allows for rapid refinement of target specificity based solely upon changes in the sequence of the miR-155 oligonucleotide mimetic. Sequence database information can be used to identify and eliminate potential variants with predicted interactions with off-target mRNA sequences allowing for rapid intelligent design of miR-155 mimetics. Furthermore, this approach also permits the potential pre-identification of non-responsive or poorly responsive subjects based on individual sequence variation within miR-155 target genes.

[0012] Such methods find particular utility in modulating one or more aspects of adipogenesis in cells and tissues of an animal, by expressing, over-expressing, under-expressing, or alternatively expressing one or more miRNA compositions, and in particular, one or more miR-155 compounds, analogs, agonists, antagonists, or a combination thereof.

[0013] Thus, in a first embodiment, the invention provides methods and compositions for diagnosing, treating, preventing, and/or ameliorating at least one or more symptoms of at least a first adipogenic-related metabolic disorder in an animal. The method generally includes at least the step of altering, affecting, stimulating, repressing, or otherwise modulating the level or extent of expression of at least one endogenous gene that is involved either in the synthesis, regulation, localization, differentiation, expression, distribution, and/or accumulation of one or more adipocytes or fatty tissues in a mammal, and in particular, humans.

[0014] Also provided herein are methods of altering, modulating, controlling, increasing, and/or attenuating at least one component, pathway, enzyme, or step involved in the process of adipocytes differentiation and/or adipogenesis activity in a cell, tissue or organ of a subject in need thereof, comprising providing to selected mammalian cells, tissues, organs, or systemically to the entire body thereof, one or more miR-155 compositions, analogs, agonists, or antagonists, or a combination thereof, in an amount and for a time effective to alter, modulate, control, increase, and/or attenuate at least one component, pathway, enzyme, or step involved in the process of adipocytes differentiation and/or adipogenesis in such cells, tissues, organ, or body.

[0015] Further provided herein are methods of treating, preventing, and/or ameliorating at least one symptom of an miR-155-associated condition, such as adipocyte expression and/or differentiation. Further provided herein are methods for controlling the rate, extent, and/or expression of at least a first adipogenic factor (or a gene encoding, expressing, or regulating such a factor) in one or more cells and/or tissues of a mammal. In certain embodiments, the method comprises administration to such cells and/or tissues a first miR-155 compound, or an antagonist, agonist, homolog, analog, inducer, or inhibitor thereof in an amount and for a time effective to alter fat synthesis, fat accumulation, fatty tissue formation, and/or fat localization within or about the body of an animal.

[0016] Further provided herein are methods of treating, preventing, and/or ameliorating at least one symptom of an miR-155-associated condition that involves expression of one or more macrophages in the cells or tissues of an animal. In certain embodiments, miR-155 compounds may be utilized to control, prevent, permit, or facilitate either the accumulation or the destruction of one or more macrophage populations. In such embodiments, the method generally involves administration of at least a first miR-155 compound, antagonist, agonist, derivative or analog thereof in an amount and time sufficient to alter the expression or modulate the activity of one or more macrophage populations. While the methods are likely to provide benefit for a variety of macrophage-related conditions, localized delivery to only select cells and/or tissues (as opposed to systemic delivery) of the compounds are more preferable in that only a particular subset of the total complement of macrophages be affected by the treatment. Such is particularly contemplated to be desirable when localized destruction or inactivation of particular macrophage populations within the body of an animal is sought.

[0017] In related aspects, miR-155 compounds may be utilized to diminish the activity of, or bring about the destruction of particular types of macrophages, either in a targeted (*i.e.*,

localized), or a generalized (*i.e.*, systemic) administration. In such embodiments, the method generally involves administration of at least a first miR-155 compound, antagonist, agonist, derivative or analog thereof in an amount and time sufficient to diminish or inhibit the activity of one or more types of macrophages within the body of an animal selected to receive the treatment.

[0018] As described herein, methods are also provided for modulating the expression of one or more miR-155-activatable or miR-155-repressible gene(s) involved in one or more steps related to adipogenesis in a mammal. In certain embodiments, the modulation of adipogenesis may be achieved by contacting a population of target cells and/or tissues with an effective amount of miR-155 composition, a homolog or analog thereof, or at least one agonist or antagonist thereof for a time sufficient to affect, alter, reduce, or modulate at least one pathway involved in mammalian adipogenesis and/or the synthesis, localization, or accumulation of fat cells or fatty tissues within or about the body of such a mammal.

[0019] In certain embodiments, the modulation comprises increasing the expression of one or more of the target genes and/or increasing the synthesis and/or accumulation of one or more gene products derived therefrom. In other embodiments, the modulation comprises decreasing the expression of one or more such target gene(s), and/or decreasing the synthesis and/or accumulation of one or more gene products derived therefrom.

[0020] Accordingly, in light of the present teachings, miR-155 compositions can now be designed that selectively modulate the expression of one or more genes (or one or more polypeptide products produced therefrom) involved in adipocyte differentiation, or in regulating the activity of one or more adipogenesis-related metabolic pathways in a mammal. Likewise, the administration of miR-155 analogs, or antagonists of one or more enzymes that bind miR-155 molecules, can now be developed to interfere with, or inhibit the activity of, one or more cellular components that control, regulate, express, or target miR-155 nucleic acids, and thus alter or attenuate one or more genes involved in the expression, localization, stability, and/or diversity of adipocytes, adipocytic precursors, and/or the accumulation of fat cells within the body of an animal, or within at least a first mammalian cell comprised within a population of isolated mammalian cells. By localizing the delivery of such miR-155 compositions to discreet parts of the body, or to particular cell or tissue types, targeted miR-155-based therapies offer particular treatment and/or prophylaxis methodologies that were heretofore unavailable to the medical community. Exemplary targeted methods afforded by the disclosed miR-155-related compounds include the ability to reduce fat cell accumulation in discreet areas of the body

without affecting generalized systemic fat cell distribution. Such methodologies may, for example, be used to (1) reduce fat cell expression in organs where such accumulation has adverse effects on health or appearance, including, for example, the liver and such like, (2) provide localized dermal contouring (including, for example, in conjunction with, or *in lieu* of, traditional liposuction modalities, *etc.*), (3) alter, regulate, or control the accumulation of fat cells in particular regions of the body undergoing localized miR-155-based therapy, or (4) facilitate cessation of, substantial reduction of, or suppression of, at least one endogenous adipogenesis enzyme, precursor, product, pathway, or such like either *ex vivo*, *in situ*, or *in vivo*.

2.1 MIR-155 COMPOSITIONS

[0021] In the practice of the invention, exemplary miR-155 composition may comprise, consist essentially of, or alternatively consist of, an oligonucleotide of from 12 to about 30 nucleotides in length that are substantially identical to, substantially complementary to, or alternatively, substantially-homologous to one or more of the specific oligonucleotides enumerated in Example 5.3 herein. Such compositions include, without limitation, an oligonucleotide that comprises a primary nucleotide sequence region that is at least 80% identical to, or at least 80% complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, as defined herein.

[0022] Such miR-155-specific compositions also further include, without limitation, an oligonucleotide of from 12 to about 30 nucleotides in length that comprises a primary nucleotide sequence region that is at least 85% identical to, or at least 85% complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, as defined herein.

[0023] Likewise, miR-155-specific compositions that comprise an oligonucleotide of from 12 to about 30 nucleotides in length that comprises a primary nucleotide sequence region that is at least 90% identical to, or at least 90% complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, as defined herein are also important aspects of the invention.

[0024] In certain embodiments, miR-155-specific compositions that comprise an oligonucleotide of from 12 to about 30 nucleotides in length that comprises a primary nucleotide sequence region that is at least 95% identical to, or at least 95% complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, also define important aspects of the invention, as do those miR-155-specific compositions that

comprise an oligonucleotide of from 12 to about 30 nucleotides in length that comprises a primary nucleotide sequence region that is at least 98% identical to, or at least 98% complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, as defined herein.

[0025] In certain embodiments, miR-155-specific compositions that comprise an oligonucleotide of from 12 to about 30 nucleotides in length that comprises a primary nucleotide sequence region that is identical to, or fully complementary to, any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, also represent illustrative aspects of the invention.

2.2 TARGETING OF MIR-155 COMPOSITIONS

[0026] The microRNA or anti-microRNA compositions of the invention may be introduced into a cell by any method known to those ordinary-skilled in the art. For example, the compositions can be injected directly into a particular cell or tissue, such as by micro miR-155 antisense, agonist, and antagonist, or alternatively, by a delivery system such as liposomes, microsomes, lipid particles and the like. Other methods for introducing a microRNA composition as described herein into one or more cells or tissues include, for example, without limitation, the use of delivery vehicles (including *e.g.,* without limitation, dendrimers, biodegradable polymers, and biocompatible polymers of amino acids or sugars, and the like, or any combination thereof).

[0027] Targeting of microRNA or anti-microRNA compositions to one or more particular cell types, tissues, and/or organs, may also be accomplished by any method known to those of ordinary skill in the art, including, for example, antibody-, antigen binding fragment- and/or ligand-specific methods. In certain embodiments, the targeting of the disclosed compositions to one or more particular cell(s) or tissue type(s) may be accomplished using one or more of the methods described in commonly-owned U.S. Prov. Patent Appl. Nos. 61/095,197 and 61/166,613, the entire contents of each of which is specifically incorporated herein in its entirety by express reference thereto.

[0028] Use of one or more of the disclosed miRNA compositions in the manufacture of a medicament for diagnosis, prophylaxis, therapy, or amelioration of at least a first symptom of a disease, disorder, abnormal condition, or metabolic state is also provided, and in particular, use of one or more such compositions in the manufacture of a medicament for preventing, treating, reducing, or ameliorating one or more symptoms of a disease, dysfunction, or disorder,

in an animal such as, without limitation, diabetes, obesity, eating disorders, excessive body weight gain, fat accumulation, hyperlipidemia, hypolipidemia, proliferative disease, neurological disease, cardiovascular disease, kidney or renal disease, liver or hepatic disease, lung or pulmonary disease, gastrointestinal disease, endocrinological disease or dysfunction, organ failure, stroke, ischemia, infarction, infection, or trauma, or any such combination thereof.

[0029] The invention also provides a method for delivering a miRNA composition to a first cell in an animal, which comprises providing to an animal in need thereof a therapeutically- or diagnostically-effective amount of one of the miRNA compositions disclosed herein.

[0030] The methods of the present invention are particularly useful in improving patient outcomes over currently practiced therapies by more effectively providing an effective amount of the selected therapeutic to populations of cells or one or more tissue sites within the body of an animal. In certain circumstances, the present invention may diminish unwanted side effects of conventional therapy, or may overcome the deleterious effects that may result from a systemic treatment modality.

2.3 THERAPEUTIC, PROPHYLACTIC AND DIAGNOSTIC COMPOSITIONS

[0031] The miRNA compositions and formulations comprising them that may be employed in the practice of the invention as a single therapeutic modality, or alternatively be combined with one or more additional therapeutic, diagnostic, and/or prophylactic agents, including, without limitation, one or more proteins, peptides, polypeptides (including, without limitation, enzymes, antibodies, antigens, antigen binding fragments *etc.*); RNA molecules (including, without limitation, siRNAs, iRNAs, mRNAs, tRNAs, and catalytic RNAs, such as ribozymes, and the like), DNA molecules (including, without limitation, oligonucleotides, polynucleotides, genes, coding sequences (CDS), introns, exons, plasmids, cosmids, phagemids, baculovirus, vectors [including, without limitation, viral vectors, virions, viral particles and such like]); peptide nucleic acids, detection agents, imaging agents, contrast agents, detectable gas, radionuclides, or such like, and pharmaceutically-active molecules, including, without limitation, one or more drugs, pro-drugs, cofactors, ligands, hormones, steroids, targeting domains, linkers, binding domains, catalytic domains, *etc.*, or any combination thereof.

[0032] The miRNA compositions of the invention may also further optionally include one or more additional active ingredients, including, without limitation, one or more adipogenic

agents, one or more anti-adipogenic agents, one or more antineoplastic or cytotoxic agents, one or more transcription factors, immunomodulating agents, immunostimulating agents, neuroactive agents, antiflammatory agents, chemotherapeutic agents, antilipidemic agents, hormones, trophic factors, cytokines, receptor agonists or antagonists, antimicrobial agents (including, without limitation, antibacterials, antifungals, antimycotics, antiamebics, antihelminthics, antivirals, and the like), antiinfective agents, or such like, or any combination thereof.

[0033] The miRNA compositions of the present invention may also further optionally include one or more liposomes, microbubbles, lipid particles, lipid complexes, or a lipid compound including, but not limited to, those selected from the group consisting of cephalin, ceramide, cerebroside, cholesterol, diacylglycerol, diacylphosphatidylglycerol diacylphosphatidylcholine, diacylphosphatidylethanolamine, phosphatidylethanolamine, phosphatidylcholine, phosphatidylethanolamine, sphingolipid, sphingomyelin, tetraether lipid, or any combination thereof, and may further optionally include one or more binding agents, cell surface active agents, surfactants, lipid complexes, niosomes, ethosomes, transferosomes, phospholipids, sphingolipids, sphingosomes, or any combination thereof, and may optionally be provided within a pharmaceutical formulation that includes one or more nanoparticles, microparticles, nanocapsules, microcapsules, nanospheres, microspheres, or any combination thereof.

[0034] Preferably, the compounds of the present invention (including for example, miR-155 antisense molecules, agonists and antagonists of miR-155 or to one of its molecular target s, as well as miR-155 analogs, derivatives, and homologous interfering RNA molecules will generally be formulated for systemic and/or localized administration to an animal, or to one or more cells or tissues thereof, and in particular, will be formulated for systemic and/or localized administration to a mammal, or to one or more cells, tissues, or organs thereof. In certain embodiments, the compounds and methods disclosed herein will find particular use in the systemic and/or localized administration of one or more adipogenic or antiadipogenic miR-155 compositions as described herein to one or more cells or tissues of a human being.

[0035] Preferably, drug-delivery formulations disclosed herein will be at least substantially stable at a pH from about 4.2 to about 8.2, and more preferably, will be substantially stable at a pH of from about 5 to about 7.5. Preferably, the active ingredient(s) and targeted drugs will be substantially active at physiological conditions of the animal into which they are being administered.

[0036] The present invention also provides for the use of one or more of the disclosed pharmaceutical compositions in the manufacture of a medicament for prophylaxis, therapy, or for the amelioration of one or more symptoms of a disease, disorder, dysfunction, or condition, and particularly for use in the manufacture of a medicament for treating, one or more diseases, dysfunctions, or disorders linked to at least a first adipogenic or antiadipogenic gene, protein, or polypeptide in a mammal, and in a human in particular.

[0037] The present invention also provides for the use of one or more of the disclosed pharmaceutical compositions in the manufacture of a medicament for prophylaxis, therapy, treatment, or amelioration of one or more symptoms of at least a first medical condition, including, without limitation, hypolipidemia, hyperlipidemia, obesity, eating disorders, excessive body weight gain, diabetes; enzyme deficiency; hormone, cofactor, or trophic factor deficiency; cardiovascular and/or cardiocirculatory disease disorder, or dysfunction; organ disease, dysfunction, or failure; genetic disorders; congenital abnormalities, defects, or malformations; trauma; or such like, or any symptom thereof.

[0038] The present invention also provides for the use of one or more of the disclosed miR-155-derived compositions, and in particular, one or more miR-155 nucleic acid compositions in the manufacture of a medicament for the prevention of a disease, disorder, or adverse condition including, without limitation, the preparation of one or more miR-155 compounds formulated for prophylactic or therapeutic administration in the modulating, alteration, or regulation of at least a first adipogenic gene or pathway thereof. Similarly, the invention also provides methods for providing a therapeutic or prophylactic compound to a first cell in a mammal, with the method generally including providing to a mammal in need thereof, an effective amount of at least a first active ingredient (such as a miR-155 composition disclosed herein) for a time effective to provide the desired therapy, prophylaxis or diagnosis in the selected mammal.

[0039] In certain aspects of the invention, the invention provides pharmaceutical compositions to facilitate the localized delivery of a therapeutically, prophylactically, or diagnostically-effective dose of one or more miR-155 compounds to a population of host cells or to one or more tissues or tissue sites within the body of a host animal. In other preferred aspects, the population of host cells or one or more tissues is included within the body of a human, or included within at least a first *ex vivo* tissue, allograft, transplanted organ, or plurality of cells, tissues, or organ that are compatible for implantation into the body of such a human as part of a typical *ex vivo* therapy protocol or such like.

2.4 THERAPEUTIC, PROPHYLACTIC AND DIAGNOSTIC METHODS

[0040] Another important aspect of the present invention concerns methods for using the disclosed miRNA compositions for treating or ameliorating the symptoms of disease, disorder, dysfunction, or deficiency in a mammal having, suspected of having, or at risk for developing, excessive fat cell accumulation or excessive fatty tissue formation. Such methods generally involve administering to a mammal (and in particular, to a human in need thereof), one or more of the disclosed miR-155 compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a disease, dysfunction, or deficiency in the affected mammal. The methods may also encompass one or more prophylactic treatment of animals suspected of having one or more such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of one or more symptoms associated with fat accumulation, obesity, body weight gain, or such like.

[0041] In certain embodiments, the therapy or prophylaxis may be provided to the animal as a single treatment modality, as a single administration, or alternatively provided to the patient in multiple administrations over a period of from several days to several weeks, to several months even to several years or longer. In some aspects, it may be desirable to continue the treatment throughout the lifetime of the patient. In other embodiments, it may be desirable to provide the miR-155-based therapy in combination with one or more existing, or conventional, treatment regimens for obesity and/or excessive fat accumulation. Examples of such combinational therapies include, for example, administration of the compositions of the present invention in combination with an exercise regimen and/or the adoption of a lower- or low-fat diet to assist in controlling overall calorie intake.

2.5 THERAPEUTIC AND DIAGNOSTIC KITS

[0042] Kits including one or more of the disclosed miRNA compositions; and instructions for using the kit in a therapeutic, diagnostic, and/or other clinical embodiment also represent preferred aspects of the present disclosure. Such kits may further include one or more of the disclosed miRNA compounds, either alone, or in combination with one or more additional therapeutic compounds, pharmaceuticals, and such like.

[0043] The kits of the invention may be packaged for commercial distribution, and may further optionally include one or more delivery devices adapted to deliver the miRNA

composition(s) to an animal (*e.g.*, syringes, injectables, and the like). Such kits may be therapeutic kits for treating, preventing, or ameliorating the symptoms of a disease, deficiency, dysfunction, and/or injury, and may include one or more miR-155 compositions of the invention, and instructions for using the kit in a therapeutic, prophylactic and/or diagnostic regimen.

[0044] The container for such kits typically includes at least one vial, test tube, flask, bottle, syringe or other container, into which the pharmaceutical composition(s) may be placed, and preferably suitably aliquotted. Where a second pharmaceutical is also provided, the kit may also contain a second distinct container into which this second composition may be placed. Alternatively, the plurality of pharmaceutical compositions disclosed herein may be prepared in a single mixture, such as a suspension or solution, and may be packaged in a single container, such as a vial, flask, syringe, catheter, cannula, bottle, or other suitable single container.

[0045] The kits of the present invention may also typically include a retention mechanism adapted to contain or retain the vial(s) or other container(s) in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vial(s) or other container(s) may be retained to minimize or prevent breakage, exposure to sunlight, or other undesirable factors, or to permit ready use of the composition(s) included within the kit.

2.6 USE OF MIR-155 INHIBITORS TO ACHIEVE FAT ACCUMULATION

[0046] While the administration of miR-155 compounds and analogs thereof is largely contemplated to be most useful in the *reduction* of adipogenesis, and hence in the reduction of fat accumulation in the body, the discoveries by the present inventors have also suggested a role for *anti*-miR-155 compounds (*i.e.*, compounds that lower, reduce, or inactive endogenous miR-155 activity in a cell). For example, by localized administration of one or more compounds that *inhibit* endogenous miR-155 activity in a cell, one can selectively *increase* adipogenesis in such cells, thereby *increasing* cellular accumulation of fat cells, or alternatively, can selectively stimulate the production of fat cells in discreet areas of the body without affecting overall fat cell distribution in the rest of the body. An exemplary method includes, for example, administration of a compound that alters, binds to, inactivates, or lessens the activity of endogenous miR-155 activity, for the purpose of *enhancing* fat cell expression in particular cells, including for example, cosmetic improvements (*e.g.*, lip augmentation and the like) or redistribution of fat cells or fatty deposits within the dermis (cosmetic reshaping, face-lifting,

tissue augmentation, and the like). Similar uses of providing *anti*-miR-155 compounds to facilitate targeted fat expression/accumulation include their use in facial reconstruction surgery, implant and augmentation procedures, and surgical intervention of the skin and supporting dermal tissues (*e.g.,* repair of burns, age-related skin damage, trauma, disease, genetic defects, corrective surgical procedures for disfigurements, and such like).

[0047] In such embodiments, the anti-mmiR-155 antagonist comprises an miR-155 antisense compound. In certain embodiments, the miR-155 antisense compound comprises a modified oligonucleotide consisting of about 12 to about 30 or so linked nucleosides, wherein the nucleobase sequence of the modified oligonucleotide is complementary to a sequence at least 80% identical to mature (*i.e.,* "native," or "wild-type") miR-155, precursor-mR-155 (*i.e.,* "pre-miR-155"), or a microRNA-155 derivative or homolog thereof. In certain embodiments, the nucleobase sequence of the miR-155 oligonucleotide has no more than about two mismatches to the nucleobase sequence of wild-type miR-155, or a precursor of such a native sequence (*e.g.,* pre-miR-155 and the like). In certain embodiments, the modified oligonucleotide is conjugated to a first ligand or ligand-binding domain. In certain embodiments, at least one internucleoside linkage of the modified oligonucleotide may comprise at least a first modified internucleoside linkage.

[0048] In certain embodiments, at least one nucleoside of the modified oligonucleotide comprises a modified sugar, and in others, *each* nucleoside of the modified oligonucleotide comprises a modified sugar. In certain embodiments, at least one nucleoside of the modified oligonucleotide comprises a modified nucleobase, and in other embodiments, one or more of the internucleoside linkages of the modified oligonucleotide includes a modified internucleoside linkage, such as, for example, a phosphorothioate internucleoside linkage.

2.7 PREPARATION OF MEDICAMENTS

[0049] Another important aspect of the present invention concerns methods for using the disclosed miRNA compositions, and in particular, miR-155 compositions, as well as formulations including one or more of them, in the preparation of one or more medicaments for preventing, treating or ameliorating the symptoms of various diseases, dysfunctions, or deficiencies in an animal, such as a vertebrate mammal. Use of the disclosed miR-155 compositions is also contemplated in therapy and/or prophylaxis of one or more diseases, disorders, dysfunctions, conditions, disabilities, deformities, or deficiencies, and in particular, those involving the rate, expression, localization, or specificity of one or more adipogenic or

adipogenesis-related gene products, and/or the regulation, modulation, or attenuation of one or more aspects of adipocyte differentiation in a mammal, and in humans in particular.

[0050] Such use generally involves administration to an animal in need thereof one or more of the disclosed miR-155 compositions, in an amount and for a time sufficient to prevent, treat, lessen, or ameliorate one or more of a disease, disorder, dysfunction, condition, or deficiency in the affected animal, or one or more symptoms thereof.

[0051] Compositions including one or more of the disclosed pharmaceutical formulations that include at least one miR-155 compositions also form part of the present invention, and particularly those formulations that further include at least a first pharmaceutically-acceptable excipient for use in the therapy, prophylaxis, or diagnosis in a mammal, and in humans, in particular.

[0052] Such formulations may optionally further include one or more additional active ingredients, detection reagents, vehicles, additives or adjuvants, radionuclides, gases, or fluorescent labels as may be suitable for administration to an animal. Such routes of administration are known to and may be selected by those of ordinary skill in the art, and include, without limitation, delivery devices including intramuscular, intravenous, intra-arterial, intrathecal, intracavitary, intraventricular, subcutaneous, or direct delivery, administration, and/or injection into an organ, tissue site, or population of cells in the recipient animal.

[0053] The use of one or more of the disclosed miR-155 compositions in the manufacture of a medicament for prophylaxis or therapy of one or more medical conditions is also an important aspect of the invention. Formulation of such compositions for use in administration to an animal host cell, and to a mammalian host cell in particular, is also provided by the invention. In particular embodiments, the invention provides for formulation of such compositions for use in administration to a human, or to one or more selected human host cells, tissues, organs *in situ*, or to an *in vitro* or *ex situ* culture thereof.

[0054] The present invention also provides for the use of one or more of the disclosed miRNA compositions in the manufacture of a medicament or a vaccine for the prophylaxis or prevention of one or more diseases or conditions, including the preparation of one or more vaccines suitable for prophylactic administration to prevent or ameliorate one or more diseases, disorders, or conditions.

[0055] The invention also provides methods for providing a therapeutic or prophylactic amount of an miR-155 compound to at least a first population of cells or to one or more tissues within the body of a mammal, with the method generally including providing to a mammal in

need thereof an effective amount of a miR-155 composition as disclosed herein and for a time effective to provide the desired therapy and/or prophylaxis in the selected cells or tissue of the mammal.

[0056] In certain aspects, the invention provides pharmaceutical compositions, and formulations thereof, that are suitable for administration to one or more mammalian host cells. In certain embodiments, the mammalian host cells are preferably human host cells. In other preferred aspects, the host cell is included within the body of a human (*i.e., in situ*), or included within at least a first *ex vivo* tissue or plurality of cells that are compatible for implantation into the body of such a human as part of an *ex vivo* therapy protocol or such like.

[0057] The pharmaceutical compositions of the present invention may be administered to a selected animal using any of a number of conventional methodologies, including, without limitation, one or more of parenteral, intravenous, intraperitoneal, subcutaneous, transcutaneous, intradermal, subdermal, transdermal, intramuscular, topical, intranasal, or other suitable route, including, but not limited to, administration, by injection, diffusion, electroporation, sonophoresis, transdermal delivery, insertion, inhalation, insufflation, or ingestion, or any combination thereof as may be medically indicated.

[0058] Yet another advantage of the present invention may include active ingredient(s) and pharmaceutical formulations and compositions that include one or more of such active ingredients useful in treating or ameliorating one or more symptom(s) of a disease, disorder, dysfunction, or abnormality in a mammal involving one or more adipogenic pathways, precursors, enzymes, products, or such like. Such methods generally involve administration to a mammal, and in particular, to a human, in need thereof, one or more of the disclosed miR-155 compositions, in an amount and for a time sufficient to treat, ameliorate at least one symptom of, or lessen the severity, duration, or extent of, such a disease, disorder, dysfunction, or abnormality in such a mammal.

[0059] The methods and compositions of the invention may also be used in prevention, prophylaxis, and/or vaccination of an animal that has, is suspected of having, is at risk for developing, is expressing one or more clinical symptoms, or has been diagnosed as potentially having one or more disorders or diseases, either before, during, or after diagnosis or the onset of one or more symptoms of the disorder or disease, or one or more clinical indicia or manifestations of at least a first symptom thereof.

3. BRIEF DESCRIPTION OF THE DRAWINGS

[0060] For promoting an understanding of the principles of the invention, reference will now be made to the embodiments, or examples, illustrated in the drawings and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended. Any alterations and further modifications in the described embodiments, and any further applications of the principles of the invention as described herein are contemplated as would normally occur to one of ordinary skill in the art to which the invention relates.

[0061] The following drawings form part of the present specification and are included to demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

[0062] **FIG. 1** shows the hierarchical clustering of miRNAs displaying altered expression during 3T3-L1 adipogenesis. Regulated miRNAs, defined as those miRNAs whose expression changed more than 2-fold at any time point during 3T3-L1 adipogenesis *vs.* basal expression, were analyzed by hierarchical cluster analysis using Cluster 3.0 software to identify miRNAs with similar expression patterns during adipocyte differentiation (Van Huissteden, 1990). This analysis identified five clusters among the 42 genes that miRNAs that were differentially expressed between the four time points of adipocyte differentiation (days 0, 1, 3 and 8 post-induction). Changes in miRNA expression are colorimetrically-represented as the log2 ratio of miRNA expression *vs.* day0 miRNA expression, as indicated at the bottom left, with the data arranged so miRNA expression at each time point is grouped within a column and miRNA expression of individual miRNAs is presented in a row. Brackets to the right indicate clusters that appear unique to different designated times. Based on the miRNA expression patterns, these 5 clusters were defined as early induction, gradual induction, late induction, gradual repression, and late repression. No miRNAs corresponding to an early repression group (day 1 peak repression) were detected among any of the miRNAs changed more than 2-fold during early adipogenesis;

[0063] **FIG. 2** illustrates a comparison of miRNA expression results as measured by microarray and RT-PCR analyses. In order to validate microarray data using quantitative RT-PCR data, 14 miRNAs that were regulated at least 2-fold at one or more points during adipocyte differentiation were analyzed to measure their relative expression at all four differentiation time points (days 0, 1, 3, and 8 post-induction). Microarray and RT-PCR

miRNA expression at each time point were normalized against day 0 expression to reformat all data as a scale-free expression ratio, graphed on a scatter-plot, and then analyzed to determine the correspondence of microarray and RT-PCR expression ratios. This analysis revealed a strong correlation between microarray and RT-PCR measured miRNA expression (Pearson $r = 0.88$; $p < 0.0001$);

[0064] **FIG. 3A and FIG. 3B** illustrate retroviral overexpression of miR-155 attenuates 3T3-L1 adipogenesis. Stable 3T3-L1 preadipocyte cell lines were infected 3T3-L1 preadipocyte cell cultures with puromycin-selectable recombinant pBABE-puro retrovirus constructs overexpressing mouse miR-155, miR-218, miR-221, or miR-222 DNA inserts or pBABE-puro retrovirus, and then cultured under puromycin selection to establish stable cell lines. FIG. 3A shows RT-PCR analysis of miRNA expression in stable 3T3-L1 preadipocyte cell lines overexpressing miR-155, miR-218, miR-221, or miR-222. RT-PCR-determined miRNA expression levels were normalized against U6 snRNA expression within each sample as a sample loading control for each amplification. Retrovirus-mediated miRNA expression values were normalized against miRNA expression in samples infected with pBABE-puro control virus, to as a control for retroviral background effects. All miRNA were expressed > 4-fold above background. FIG. 3B shows overexpression of miR-155 inhibits 3T3-L1 preadipocyte cell differentiation. Stable 3T3-L1 preadipocyte cell lines carrying control pBABE-puro retrovirus (Ctrl) or retroviruses overexpressing miR-155, miR-218, miR-221, or miR-222 were induced to differentiate with adipocyte differentiation media, cultured for 8 days and then stained with Oil Red O to detect lipid accumulation as a marker of adipocyte differentiation. Results shown indicate triplicate wells of each 3T3-L1 preadipocyte cell lines after culture, differentiation and Oil Red O staining on a 96-well plate. Note that the miR-155 overexpressing 3T3-L1 preadipocyte cell line demonstrated a marker decrease in adipocyte differentiation;

[0065] **FIG. 4A, FIG. 4B, and FIG. 4C** demonstrate that overexpression of miR-155 in 3T3-L1 cells suppresses adipogenesis. FIG. 4A shows 3T3-L1 preadipocyte cell lines carrying stable retroviral integrants of pBABE-puro or pBABE-puro overexpressing miR-155 were induced to differentiate with adipocyte differentiation medium, cultured for 8 days, then stained with Oil Red O to detect lipid accumulation as a marker of adipocyte differentiation. Note that adipocyte differentiation as determined by Oil Red O staining was markedly attenuated in 3T3-L1 preadipocytes overexpressing miR-155. FIG. 4B illustrates RT-PCR analysis of adipocyte marker gene expression at 0, 1, 3, 6, and 9 days after induction of adipocyte differentiation in

3T3-L1 pBABE-puro and pBABE-puro preadipocyte cell lines. RT-PCR-measured FABP4, C/EBPα and PPARγ gene expression was normalized against 18S RNA expression as a reference control, and then normalized against day 0 gene expression in pBABE-puro 3T3-L1 cells in order to present data as fold-change *vs.* the un-induced preadipocyte control baseline. Error bars indicate standard deviation of the mean. FIG. 4C illustrates a Western blot analysis of adipocyte marker gene protein expression at 0, 2, 4, 6, and 8 days after induction of adipocyte differentiation in 3T3-L1 pBABE-puro and pBABE-puro preadipocyte cell lines. Note that there is a marked decrease in the adipocyte marker proteins FABP4, C/EBPα and PPARγ, but no difference in the cytoskeletal protein, β-actin, which was used as a loading control;

[0066] **FIG. 5A and FIG. 5B** depict transfection of 3T3-L1 preadipocytes with a miR-155 inhibitor oligonucleotide promotes adipogenesis. FIG. 5A shows 3T3-L1 preadipocytes transfected with a miR-155 inhibitor *vs.* a scrambled negative control oligonucleotide demonstrate increased Oil Red O staining 6 days after induction of adipogenesis. FIG. 5B shows 3T3-L1 cells transfected with a miR-155 inhibitor oligonucleotide *vs.* control oligonucleotide reveal increased expression of adipocyte marker genes 6 days after induction of adipogenesis. Error bars indicate standard deviation of the mean;

[0067] **FIG. 6** shows the miR-155 expression pattern during 3T3-L1 adipogenesis. Note that the time scale is not linear. Data are presented as means ± SE fold induction relative to miR-155 expression at day 0. Data are presented as means ± SE of the mean; n = 3/group;

[0068] **FIG. 7A and FIG. 7B** show retroviral overexpression of miR-155 in 3T3-F442A cells suppresses insulin-induced adipogenesis. FIG. 7A shows 3T3-F442A preadipocyte cell lines carrying stable retroviral integrants of pBABE-puro or pBABE-puro overexpressing miR-155 were induced to differentiate with 10 μg/mL insulin, cultured for 12 days, then stained with Oil Red O to detect lipid accumulation as a marker of adipocyte differentiation. FIG. 7B illustrates RT-PCR analysis of adipocyte marker gene expression 10 days after induction of adipocyte differentiation. Note that adipocyte differentiation, as assayed by both Oil Red O staining and FABP4 and PPARγ mRNA expression, was markedly attenuated in 3T3-L1 preadipocytes overexpressing miR-155;

[0069] **FIG. 8A and FIG. 8B** show RT-PCR miR-155 expression profile in the tissues of adult C57BL/6J mice. FIG. 8A shows mice differentially express miR-155 in a broad variety of tissues. RT-PCR analysis detected miR-155 expression in white adipose tissue (WAT), brown adipose tissue (BAT), heart, white blood cells (WBC; peripheral blood

mononuclear cells), brain, bone marrow (BM), intestine, kidney, liver, lung, skeletal muscle (SkM; gastroc), pancreas, spleen, stomach, and aorta (vessel). FIG. 8B shows expression of miR-155 in adipose tissue fractions. RT-PCR analysis indicates that miR-155 is expressed in both mature adipocytes (Ads) and the stromovascular fraction cells (SVF: preadipocytes, endothelial cells, vascular smooth muscle cells, macrophages, *etc.*). RT-PCR miR-155 values are normalized against snoRNA202 expression. Data are presented as means ± SE of the mean; n = 3/group;

[0070] **FIG. 9** demonstrates that the C\EBPβ 3′-UTR contains an evolutionarily conserved miR-155 binding site. An evolutionarily-conserved putative miR-155 binding site in the mouse C/EBPβ 3′-UTR (439-461 nucleotides 3′ of the mouse C/EBPβ stop codon)(SEQ ID NO:16), contains the 8 nucleotide 'seed sequence' essential for miR-155 interaction with mRNA (shaded area). Note that there is also substantial conservation of nucleotides capable of forming canonical and wobble base pairs (guanine to uracil) with the remaining miR-155 sequence alignment: Human (SEQ ID NO:17); Rat (SEQ ID NO:18); Dog (SEQ ID NO:19) and Chicken (SEQ ID NO:20). The alignment is shown against the murine sequence for miR-155 (SEQ ID NO:15);

[0071] **FIG. 10A and FIG. 10B** show retroviral overexpression of miR-155 decreases C/EBPβ expression. Replica wells of 3T3-L1 preadipocyte cell lines carrying stable retroviral integrants of pBABE-puro or pBABE-puro overexpressing miR-155 (miR-155) were induced to differentiate with 3T3-L1 adipocyte differentiation medium, and (FIG. 10A) harvested to isolate RNA for RT-PCR analyses at 0, 2, 4, 10, and 24 hours post-induction or (FIG. 10B) harvested to isolate protein for Western blot analyses at 0, 12, 24, and 48 hours post-induction. In FIG. 10A, both C\EBPβ and C\EBPα mRNA expression was significantly reduced at baseline (0 hour), but only C\EBPα mRNA expression was remained significantly attenuated at all times post-induction. In FIG. 10B, C\EBPβ and C\EBPα protein expression, normalized against β-actin expression in the same samples in order to correct for loading variations, is presented below a representative Western blot of each experiment. C\EBPβ protein expression was significantly repressed in miR-155 overexpressing cells, and remained significantly repressed over the first 24 hours post-induction, while C\EBPα protein expression was significantly reduced by 12 hours post-induction and remained attenuated out to 48 hours post-induction. Data are presented as means ± SE (n = 3/group; *$p < 0.05$ *vs.* pBABE-puro by Student's *t*-test);

[0072] **FIG. 11A, FIG. 11B, FIG. 11C, and FIG. 11D** show C\EBPβ is a direct target of miR-155. Transfection of 3T3-L1 preadipocytes with a miR-155 mimic represses (FIG. 11A) C/EBPβ mRNA and (FIG. 11B) C/EBPβ protein expression. Both C/EBPβ and C/EBPα mRNA expression was reduced in cells transfected with miR-155 mimic, but neither C/EBPβ nor C/EBPα mRNA expression was significantly altered in cells transfected with miR-155 inhibitor. C/EBPβ and C/EBPα mRNA expression in all samples is normalized against that of the 3T3-L1 samples transfected with the mimic oligonucleotide negative-control (NC). β-actin protein was assayed as a loading control for protein analysis of miR-155 mimic and inhibitor effects on C/EBPβ and C/EBPα protein expression. FIG. 11C shows luciferase activity of reporter constructs carrying wild-type C/EBPβ 3′-UTR sequence (C/EBPβ-wt) is inhibited by co-transfection with miR-155 mimic, but not by co-transfection with irrelevant miR-27 or NC mimics. This inhibition requires an evolutionarily-conserved wild-type C/EBPβ 3′-UTR miR-155 target sequence, since miR-155 mimic co-transfection had no effect in cells transfected with luciferase constructs containing a site-specific mutation of the C/EBPβ 3′-UTR miR-155 target site (C/EBPβ-mut); FIG. 11D shows luciferase activity of reporter constructs carrying wild-type C/EBPα 3′-UTR sequence is not inhibited by co-transfection with miR-155 mimic, but is efficiently silenced by a C/EBPα-specific siRNA;

[0073] **FIG. 12A and FIG. 12B** show C\EBPβ overexpression partially rescues adipogenesis in 3T3-L1 preadipocyte overexpressing miR-155. 3T3-L1 preadipocyte cell lines were infected as indicated with either empty retrovirus (pBABE-puro) and/or retrovirus overexpressing miR-155, C/EBPβ or C/EBPα, induced to differentiate, and either (FIG. 12A) stained with Oil-Red O after to detect lipid accumulation as a marker of adipogenesis after 6 days of differentiation or (FIG. 12B) harvested at 0, 6, 12, 24, or 48 hours post-inducted for Western-blot of C/EBPα expression. β-actin expression was analyzed on duplicate blots generated at the same time to detect sample variations. Note that C/EBPα expression is not significantly induced in 3T3-L1 cells overexpressing C/EBPβ.

[0074] **FIG. 13A, FIG. 13B, and FIG. 13C** show epididymal white adipose tissue miR-155 expression is increased in three different mouse models of obesity. FIG. 13A shows male wild-type and Agouti (A^y) and FIG. 13B shows male db/m and db/db mice that were fed standard chow diet until sacrificed for tissue collection at 6- and 3-months of-age, respectively (n = 4/group). In FIG. 13C, male low-density lipoprotein receptor deficient ($LDLR^{-/-}$) mice were fed standard chow diet or high-fat Western diet for 12 weeks, starting at 10 weeks of-age,

then sacrificed for tissue collection (n = 6/group). RT-PCR data for epididymal white adipose tissue miR-155 expression are normalized against U6 snRNA expression within the same sample, and miR-155 expression in the obese mouse models (A^y, db/db, HFD-fed $LDLR^{-/-}$) are normalized against their lean controls (wild-type, db/m, chow-fed $LDLR^{-/-}$). Data are presented as means ± SE; *p < 0.05 *vs.* lean control by Student's *t*-test;

[0075] **FIG. 14A and FIG. 14B** show SVF cells and preadipocytes of obese vs. lean mice have increased miR-155 expression. Male db/m and db/db male mice fed standard chow diet and sacrificed at 3-months-of-age for adipose isolation. Male $LDLR^{-/-}$ mice were fed standard chow diet or high-fat Western diet for 12 weeks, starting at 10 weeks of age, the sacrificed for adipose tissue isolation. RT-PCR data of SVF, adipocyte and preadipocyte expression from (FIG. 14A) db/m and db/db and (FIG. 14B) chow- and HFD-fed $LDLR^{-/-}$ mice. Sample miR-155 expression values were normalized against U6 snRNA expression in the same sample. Data are presented as means ± SE; A: SVF (6/group), adipocyte (5-6/group), preadipocyte (3/group); B: SVF (4-5/group), adipocyte (4-5/group), preadipocyte (3/group); *$p < 0.05$ *vs.* matching lean control sample by Student's *t*-test;

[0076] **FIG. 15A and FIG. 15B** show RT-PCR analysis for the relative depletion of macrophages and endothelial cells from the preadipocyte fraction. SVF and preadipocyte cell fractions isolated from db/m and db/db epididymal white adipose tissue, and analyzed in FIG. 14A and FIG. 14B, were RT-PCR analyzed for expression of (FIG. 15A) CD68, a macrophage marker, and (FIG. 15B) PECAM-1, an endothelial cell marker, in order to assess the relative expression of these genes in SVF and preadipocyte fractions. RT-PCR analysis revealed strong reductions in both CD68 (1% SVF) and PECAM-1 (0-3% SVF) *vs.* the SVF source material, strongly suggesting that miR-155 expression differences in db/m and db/db preadipocyte fractions do not arise from differential macrophage or endothelial cell contributions. CD68 and PECAM-1 mRNA expression values were normalized against 18S rRNA expression in the same sample. Data are presented as means ± SE; SVF (n = 4/group), preadipocyte (n = 3/group); *$p < 0.005$ *vs.* matching SVF sample by Student's *t*-test;

[0077] **FIG. 16A and FIG. 16B** show TNFα increases 3T3-L1 preadipocyte miR-155 expression in a dose- and time-dependent manner. FIG. 16A shows time course of miR-155 expression in 3T3-L1 preadipocytes after exposure to 2 ng/mL TNFα. FIG. 16B shows dose-response curve of 3T3-L1 preadipocyte miR-155 expression after 24 hours exposure to 0, 0.5, 1, 2, or 5 ng/mL TNFα. Sample miR-155 expression values were normalized against U6 snRNA expression in the same sample. Data are presented as means ± SE; n = 2/group;

[0078] **FIG. 17** shows 3T3-L1 preadipocyte miR-155 expression was increased by exposure to several different inflammatory cytokines. 3T3-L1 preadipocytes were treated for 24 hours with TNFα (2 ng/mL), LPS (200 ng/mL), IL-1β (5 ng/mL), IFNγ (2 ng/mL), IL-6 (5 ng/mL), or TGFβ (5 ng/mL), or a combination (IFs) of TNFα (2 ng/mL), IL-1β (5 ng/mL), and IFNγ (2 ng/mL), harvested for total RNA, and RT-PCR analyzed for miR-155 expression. Data are presented as means ± SE; n = 4/group; *$p < 0.005$ *vs.* matching SVF sample by Student's *t*-test;

[0079] **FIG. 18A and FIG. 18B** show epididymal white adipose tissue and SVF fractions of obese mice increased expression of inflammatory cytokines that increase preadipocyte miR-155 expression. Male db/m and db/db male mice fed standard chow diet and sacrificed at 3-months-of-age for adipose isolation and fractionation. Male $LDLR^{-/-}$ mice were fed standard chow diet or high-fat Western diet for 12 weeks, starting at 10 weeks of age, the sacrificed for adipose tissue isolation and fractionation. RT-PCR data of pro-inflammatory gene expression in epididymal white adipose tissue (WAT) and SVF samples isolated from (FIG. 18A) db/m and db/db and (FIG. 18B) chow- and HFD-fed $LDLR^{-/-}$ mice were normalized against 18S rRNA expression in the same sample. Data are presented as means ± SE; db/m and db/db WAT and SVF (n = 4/group), $LDLR^{-/-}$ WAT and SVF (n = 4-5/group); *$p < 0.05$ *vs.* matching lean control sample by Student's *t*-test;

[0080] **FIG. 19A and FIG. 19B** show TNFα-mediated inhibition of 3T3-L1 adipogenesis is attenuated in cells transfected with miR-155 inhibitor. Replica wells of 3T3-L1 preadipocytes were transfected for 6 hours with Lipofectamine 2000 (Invitrogen) and 40 pmol of synthetic miR-155 inhibitor or control oligonucleotide (Dharmacon, Lafayette, CO, USA), then stimulated to differentiate with 3T3-L1 in the presence or absence of 2 ng/mL TNFα. Replica plates were harvested 6 days post-induction and (FIG. 19A) stained with Oil Red O to quantitate lipid accumulation or (FIG. 19B) harvested for total RNA isolation to measure expression of the adipocyte marker gene FABP4. Data are presented as means ± SE; (n = 4/group); *$p < 0.05$ *vs.* no TNFα treatment by Student's *t*-test;

[0081] **FIG. 20** shows inflammatory gene expression is increased in mature adipocytes transfected with an miR-155 mimic oligonucleotide. Replica 12-well plates of 3T3-L1 preadipocytes were induced to differentiate, then 8 days post-induction mature 3T3-L1 adipocytes were transfected for 6 hours with miR-155 mimic (miR-155) or negative control (NC) oligonucleotides, allowed to recover for 24 hours, then harvested for RT-PCR analysis of gene expression. Sample gene expression values were normalized against 18S rRNA

expression in the same sample. Data are presented as means ± SE; n = 3/group; *p < 0.05 *vs.* NC sample by Student's *t*-test;

[0082] **FIG. 21A and FIG. 21B** show C/EBPβ induces miR-155 expression. In FIG. 21A, replica 12 well plates of 3T3-L1 preadipocytes were transfected with a control siRNA, or siRNAs specific for C/EBPα, C/EBPβ, or KLF5, switched to 3T3-L1 adipocyte differentiation, and then harvested for RNA isolation for RT-PCR analysis of candidate gene expression 24 hours post-induction. Data are presented as means ± SE; (n = 4/group; *p < 0.05 *vs.* day 0 sample by Student's *t*-test. In FIG. 21B replica plates of 3T3-L1 3T3-L1 preadipocytes were co-infected with puromycin-selectable control (pBABE-puro) or miR-155-overexpressing (miR-155) retrovirus and hygromycin-selectable control (pBABE-hygro) or C/EBPβ-overexpressing (C/EBPβ) retroviruses for 14 days and then harvested to isolate total cellular RNA for RT-PCR analysis of miR-155 expression. Data are presented as means ± SE; (n = 4/group; *p < 0.05 *vs.* matching pBABE-hygro sample by Student's *t*-test;

[0083] **FIG. 22A and FIG. 22B** show mouse embryonic fibroblasts (MEFs) of isolated from miR-155 KO mice demonstrate increased adipogenesis during *in vitro* differentiation. Heterozygous miR-155 KO mice were crossed to generate wild-type, miR-$155^{+/-}$ and miR-$155^{-/-}$ embryos, and MEFs were isolated from E12.5 littermate embryos, cultured, and induced to differentiate and then replica plates were (FIG. 21A) stained with Oil Red O to detect lipid accumulation and (FIG. 21B) processed to generate total cellular RNA for RT-PCR analysis of FABP4 gene expression. Embryos were genotyped to determine the genotype of the resulting MEFs, and wild-type (WT) and miR-155 KO MEF samples were compared for differences. RT-PCR data was normalized against 18S RNA expression as a reference control, and then normalized against wild-type MEF gene expression in order to present data as fold-change *vs.* the wild-type baseline. RT-PCR data are presented as means ± SE; n = 4/group; *p < 0.05 *vs.* wild-type (WT) littermates by Student's *t*-test. Both Oil Red O and FABP4 mRNA expression were markedly enhanced in MEFs derived from miR-155 KO mice;

[0084] **FIG. 23A, FIG. 23B, and FIG. 23C** show miR-155 KO mice develop larger and more metabolically active adipose tissue depots during post-natal adipose differentiation. FIG. 23A shows body weights and NMR-determined percent body fat of 3-day-old wild-type and miR-155 KO littermate mice (n = 16/group). FIG. 23B shows representative histological images of adipocytes in the scapular region subcutaneous fat depot in 3-day-old wild-type and miR-155 KO littermate mice, and quantitation of adipocyte diameter (N=3/group). FIG. 23C shows expression of adipogenic transcription factors (C/EBPα, C/EBPβ and PPARγ) and lipid

metabolism genes (FABP4 and HSL) in epididymal WAT of 3-day-old mice (n = 4-5/group). Data are presented as means ± SE; *p < 0.05 *vs.* wild-type (WT) littermates by Student's *t*-test;

[0085] **FIG. 24A, FIG. 24B, and FIG. 24C** show adipose tissue of adult miR-155 KO mice demonstrates gene expression changes consistent with altered adipogenesis and activity. Several genes involved in A) adipogenesis and B) fatty acid storage and C) metabolism are altered in 10-week-old adult miR-155 KO mouse epididymal white adipose tissue. Adipose tissue of miR-155 KO mice had higher expression of adipogenic transcription factors and increased expression of genes expected to both increase and decrease lipolysis and fatty acid β-oxidation. RT-PCR data were normalized against 18S rRNA expression in the same sample and then normalized against wild-type gene expression. Data are presented as means ± SE; N=4-6/group; *p < 0.05 *vs.* wild-type (WT) littermates by Student's *t*-test;

[0086] **FIG. 25A and FIG. 25B** show adult miR-155 KO mouse adipose tissue reveals decreased expression of individual pro-inflammatory genes and increased expression of selected adipokines. A) CD68 expression is modestly increased in 10-week-old adult miR-155 KO mouse adipose tissue, but the expression of select pro-inflammatory genes either remain unchanged or decrease. B) Resistin, angiotensin and adiponectin expression is increased or tends to increase in RT-PCR data were normalized against 18S rRNA expression in the same sample and then normalized against wild-type gene expression. Data are presented as means ± SE; n = 4-6/group; *p < 0.05 *vs.* wild-type (WT) littermates by Student's *t*-test;

[0087] **FIG. 26A, FIG. 26B, FIG. 26C, FIG. 26D, FIG. 26E, and FIG. 26F** show genomic miR-155 deficiency alters body composition in response to high-fat diet. Adult 10-week-old Wild-type (WT) and miR-155 KO (KO) male mice demonstrated differential adipogenic responses when fed high-fat diet (HFD). Wild-type and miR-155 KO male mice had similar starting weights, but miR-155 KO mice tended to gain significantly more weight (FIG. 26A and FIG. 26B) and fat mass (FIG. 26C and FIG. 26D) than wild-type mice when fed HFD, but revealed only a transient difference in lean mass (FIG. 26E and FIG. 26F). Data are presented as means ± SE; n = 6/group); $^{+}p < 0.1$ and $*p < 0.05$ *vs.* wild-type mice by Student's *t*-test or Mann-Whitney U-test, as appropriate;

[0088] **FIG. 27A and FIG. 27B** show genomic miR-155 deficiency alters body composition in response to high-fat diet. FIG. 27A shows HFD-fed miR-155 KO mice are visibly larger than wild-type mice after 8 weeks of diet. In FIG. 27B, epididymal fat pads were significantly enlarged in HFD-fed miR-155 KO *vs.* wild-type mice, corresponding to increased

whole-body fat increases, suggesting that KO-associated fat mass increases were not fat depot specific;

[0089] **FIG. 28A, FIG. 28B, FIG. 28C, FIG. 28D, FIG. 28E, and FIG. 28F** show genomic miR-155 deficiency alters body composition in response to standard chow diet. Adult 10-week-old wild-type (WT) and miR-155 KO (KO) male mice demonstrated differential adipogenic responses when fed chow diet. FIG. 28A and FIG. 28B show wild-type and miR-155 KO male mice had similar starting weights and both groups gained similar weight when fed chow diet. However, despite the fact that wild-type and miR-155 KO mice gained similar weight when fed chow diet, miR-155 mice gained significantly less fat mass than wild-type mice (FIG. 28C and FIG. 28D), demonstrating no significant increases in fat mass from baseline after 8 weeks of diet. NMR-determined lean body mass increases in miR-155 KO *vs.* wild-type mice (FIG. 28E and FIG. 28F), which did not reach statistical significance, appeared to parallel fat mass gains in the wild-type mice, resulting in similar final weights. Data are presented as means ± SE; WT n = 6/group, KO n = 18/group; $^{+}p < 0.1$ and $^{*}p < 0.05$ *vs.* wild-type by Student's *t*-test or Mann-Whitney U-test, as appropriate;

[0090] **FIG. 29A, FIG. 29B, and FIG. 29C** show genomic miR-155 deficiency alters insulin sensitivity in response to standard chow and high fat diets. FIG. 29A shows chow-fed miR-155 KO and wild-type mice demonstrated similar insulin-sensitivity when analyzed using intraperitoneal insulin tolerance tests (ITT), while FIG.29B shows HFD-fed miR-155 KO demonstrated increased insulin resistance *vs.* their wild type controls. Data are presented as means ± SE; n = 6/group; $^{*}p < 0.05$ *vs.* wild-type. FIG. 29C shows HOMA-IR analysis of insulin sensitivity. HFD-fed miR-155 KO mice were more insulin-resistant than HFD-fed wild type mice or chow-fed KO mice, while HFD-fed wild-type mice were no more insulin-resistant than their chow-fed controls. Surprisingly, however, chow-fed miR-155 KO mice were significantly more insulin sensitive than their wild-type controls, despite similar body weights and only modest differences in body composition. Data are presented as means ± SE; n = 4-6/group; $^{*}p < 0.05$ *vs.* wild-type, and $^{\dagger}p < 0.05$ *vs.* KO chow by 1-way ANOVA);

[0091] **FIG. 30A and FIG. 30B** show synthetic miR-155 oligonucleotide mimetics induce cell death in both primary macrophages and immortalized macrophage cell lines. Transfection with a synthetic miR-155 mimic, but not its negative control (Ctrl) markedly and progressively decreased cell viability in culture of both) immortalized RAW264.7 mouse macrophages (FIG> 30A) and primary bone-marrow-derived mouse macrophages (FIG. 30B). Data are presented as means ± SE, n = 2/group;

[0092] **FIG. 31A and FIG. 31B** show evolutionary conservation of mature miR-155 murine (SEQ ID NO:15) and human (SEQ ID NO:21) sequences and the C/EBPβ 3′-UTR mir-155 target sites from a variety of species. FIG. 31A shows a sequence alignment of mature human (SEQ ID NO:21)and mouse (SEQ ID NO:15) miR-155 sequence with an evolutionarily conserved C/EBPβ 3′-UTR mir-155 target site from thirteen species (SEQ ID NO:22 through SEQ ID NO:34). An underlined uracil residue, nucleotide 12 of the miR-155 sequence, indicates the single nucleotide difference between mature human and mouse miR-155 sequence. Dashes indicate spaces introduced to maximize sequence alignment. In FIG. 31B, synthetic miR-155 compositions are depicted against a sequence alignment of mature human miR-155 and its C/EBPβ 3′-UTR target. The "Watson-Crick" sequence (SEQ ID NO:35) indicates nucleotide substitutions that would generate Watson-Crick base pairs at the indicated positions, while the "Wobble" sequence (SEQ ID NO:36) indicates the position of guanine substitutions that should produce guanine-uracil wobble base pairs. Dashes indicate non-targeted nucleotide positions. Numbers beneath the aligned sequence indicate the respective miR-155 nucleotide positions (23-1). Note that miR-155 sequence is aligned 3′ to 5′; and

[0093] **FIG.32A and FIG. 32B** show the evolutionary conservation of mature miR-155 sequences. FIG. 32A shows the linear sequence alignment of 15 mature miR-155 sequences (SEQ ID NO:1 through SEQ ID NO:5) from the University of Manchester miRBase database (Release 15; http://www.mirbase.org) with human miR-155 (SEQ ID NO:21). Red underlined text indicates nucleotide differences from a direct linear alignment with the human miR-155 sequence. FIG. 32B shows the nucleotide conservation among aligned miR-155 sequences. Nucleotide matches between a linear alignment of human miR-155 with other known miR-155 sequences are represented as dashes, while mismatches are indicated by showing the species-specific nucleotide sequence at the corresponding alignment position. Note that miR-155 sequence is aligned 5' to 3'.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0094] Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort

might be complex and time-consuming, but would be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

4.1 MICRORNA

[0095] MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate their mRNA targets by binding with imperfect complementarity in the 3'-untranslated region (see, *e.g.,* U.S. Patent 7,416,842, and Bartel, 2004, each of which is specifically incorporated herein in its entirety by express reference thereto, for a detailed review of microRNA's and methods for their synthesis, modification and/or use both *in vitro* and *in vivo*). A new class of short RNA molecules, called microRNAs (miRs), has recently been shown to play important roles in the regulation of protein expression through recognition of complementary sequences in specific target messenger RNAs. Several hundred miRs, with differing specificities, have been described to date in various vertebrates.

4.2 OBESITY AND DIABETES

[0096] Obesity is a complex disorder and often leads to hyperinsulinemia, hyperglycemia and insulin resistance; moreover, obesity is a major risk factor for hypertension and cardiovascular disease. Based on the current incidence of overweight and obesity, which now affect more than half of the adult United States population, the current lifetime risk of diabetes for 5-year-olds today is predicted to be 33% for boys and 39% for girls. Diabetes is a vascular disease that results in a 4-fold or greater increase in atherosclerotic progression, a 3-fold increased risk of heart failure, and is the most common cause of adult blindness, non-traumatic amputation, and end-stage renal disease in the US. Even in the absence of diabetes, overweight and obesity are associated with several cardiovascular risk factors, including elevated triglycerides and reduced HDL cholesterol, hypertension, and altered glucose homeostasis. Current non-invasive approaches to prevent weight gain or promote weight loss to treat these cardiovascular risk factors, which focus on changes in diet and exercise behavior, have predominantly poor results.

[0097] The present invention addresses these issues by providing compositions and methods for directly (and/or indirectly) regulating one or more steps involved in adipocyte differentiation by modulating the level of endogenous miR-155 expression, or through exogenous administration of miR-155 mimetics or inhibitors. Such methods offer several

significant improvements over the conventional methods for managing diabetes, obesity, or controlling weight loss in selected individuals.

4.3 MODIFIED MIRNAS AND OLIGONUCLEOTIDES

[0098] In certain embodiments, the invention provides oligonucleotides that comprise one or more modifications to a nucleobase, sugar, and/or internucleoside linkage.

[0099] Examples of modified oligonucleotides, and methods for their preparation are known in the art, and are exemplified *e.g.,* in U.S. Patent Appl. Nos. 2008/0300211 and 2009/0099116, each of which is specifically incorporated herein in its entirety by express reference thereto.

[00100] In certain embodiments, the methods comprise administration of a compound comprising a modified oligonucleotide. In certain embodiments, a modified oligonucleotide has a sequence that is complementary to a miRNA or a precursor thereof. In certain embodiments, the miRNA is miR-155.

4.4 MIRNA QUANTITATION ASSAYS

[00101] The effects of antisense inhibition of a miRNA following the administration of modified oligonucleotides may be assessed by a variety of methods known in the art, including, *e.g.,* without limitation, those disclosed in U.S. Patent Appl. No. 2008/0300211.

[00102] Total RNA samples used for miRNA quantitation in the indicated studies were isolated from cell culture or tissue samples using TRIzol Reagent (Invitrogen/Life Technologies Corp., Carlsbad, CA, USA) or miRNeasy Mini Kits (Qiagen, Valencia, CA, USA) and reverse-transcribed to cDNA using an Applied Biosystems TaqMan® miRNA Reverse Transcription Kits (Invitrogen/Life Technologies Corp.). Resulting cDNA samples were RT-PCR analyzed using a 7900HT Fast Real-Time PCR system (Invitrogen/Life Technologies Corp.) with miRNA-specific TaqMan® primers and probes (Invitrogen/Life Technologies Corp.). RT-PCR expression data for a given miRNA within a sample was normalized against U6 snRNA expression within the same sample measured on the same reaction plate, as a reference control.

4.5 MIRNA SYNTHESIS

[00103] Synthetic oligonucleotides functioning as miR-155 mimics or inhibitors were commercially available from Thermo Scientific Dharmacon. miRIDIAN™ microRNA mimics are double-stranded RNA oligonucleotides that have been chemically-modified to enhance performance relative to native-based miRNA mimic designs. miRIDIAN™ microRNA hairpin

inhibitors are single-stranded oligonucleotides that contain a novel secondary structure motif and chemical modifications to increase functional potency and longevity, while reducing toxicity. miRIDIAN™ microRNA negative control (NC) sequences are based on *C. elegans* miRNAs cel-miR-67 (NC1: 5′-UCACAACCUCCUAGAAAGAGUAGA-3′ [SEQ ID NO:40]; miRBase accession: MIMAT0000039) and cel-miR-239b (NC2, 5′-UUGUACUACACAAAAGUACUG-3′ [SEQ ID NO:41]; miRBase accession: MIMAT0000295), that demonstrate minimal sequence identity with known human, mouse and rat miRNAs and demonstrate little similarity to known human, mouse and rat mRNAs. Chemical and structural modifications to miRIDIAN™ microRNA mimic and hairpin inhibitor oligonucleotides are proprietary to Thermo Scientific Dharmacon.

4.6 CELL- AND TISSUE-SPECIFIC OR -SELECTIVE DELIVERY METHODS

[00104] Cell- or tissue-specific or -selective delivery therapeutic approaches are preferable in order to avoid or reduce potential deleterious effects that may result from a systemic treatment modality. Several approaches may be useful to increase the delivery specificity or selectivity of a miR-155-related therapeutic agent, targeted delivery to cells expressing cell-specific or -selective markers, directed delivery into targeted tissue sites, cell type-specific or restricted therapeutic expression, or a combination of these approaches. Cell-surface markers specific to or selective for, preadipocytes or mature adipocytes are a means of enriching delivery within a tissue or selectively targeting these cell types.

[00105] Currently, the only widely accepted preadipocyte marker is preadipocyte factor-1 (Pref-1) (Villena *et al.*, 2002). Pref-1 is a transmembrane protein containing an extracellular domain with six epidermal growth factor repeat -like repeats (Smas *et al.*, 1994), which can be converted into a biologically active soluble form through the action of TNFα converting enzyme (Wang and Sul, 2006). Pref-1 mRNA and protein levels are high in 3T3-L1 preadipocytes, but decrease during adipocyte differentiation and are absent in mature adipocytes. Pref-1 expression upregulates Sox9, which directly interacts with and represses the promoters of C/EBPβδ and C/EBPδ to block adipocyte differentiation (Wang and Sul, 2009). However, Pref-1 is also significantly expressed in several non-adipose tissues, including the placenta, pituitary, adrenal cortex, fetal liver, and pancreatic islet cells (Gesta *et al.*, 2007). Similarly, other putative cell-surface preadipocyte markers, such as collagen type VI α2 chain (COL6A2) (Hu *et al.*, 1998) and secreted frizzled-related protein 2 (SFRP2) (Gesta *et al.*, 2006), also fail to demonstrate adipose tissue specificity.

[00106] Based on the available scientific literature, a systemic delivery approach for directly targeting preadipocytes does not appear to be technically feasible. However, strategies using preadipocyte-selective markers such as Pref-1, COL6A2 or SFRP2 may be useful to selectively target preadipocytes after regional delivery of an miR-155-related therapeutic agent. Several cell surface proteins that reportedly play important roles in adipocyte function, such as adiponutrin and aquaporin 7, demonstrate selectivity for mature adipocytes. Adiponutrin, a transmembrane triglyceride hydrolase under tight nutritional control, is highly expressed in adipose tissue and markedly increases during adipogenesis but is virtually undetectable in all other tissues under normal dietary conditions (Baulande *et al.,* 2001; Huang *et al.,* 2010; Lake *et al.,* 2005). Adiponutrin is tightly regulated by caloric intake, showing strong adipose expression in fed mice, weak expression in fasted mice, and even higher expression in fasted/re-fed mice (Baulande *et al.,* 2001). However, while liver adiponutrin expression is ~1% that of adipose tissue is lean mice, exposure to a high-fat Western-type diet increase liver adiponutrin expression ~20-fold, significantly reducing the probable adipocyte-selectivity of this protein in obese subjects consuming a high-fat diet. Aquaporins form simple pores that enable water to pass bi-directionally through cell membranes according to osmotic gradients, with aquaporins 3, 7, 9, and 10 subcategorized as aquaglyceroporins that are able to allow passage of both glycerol and water (Maeda *et al.,* 2008). Aquaporin 7 is preferentially expressed in adipose, showing strong expression in both white and brown adipose and testis tissue, and more weakly expressed in cardiac, skeletal muscle and kidney tissue. Adipose tissue aquaporin 7, displays nutritional control opposite that of adiponutrin, since its gene expression is reduced in fed animals and increased in fasted animals, in parallel with plasma glycerol levels but opposite plasma insulin concentrations (Kishida *et al.,* 2000). Finally, recent reports also indicate that two other proteins, adipogenin and mouse homologue of rat prostatic androgen-repressed message-1 (mPARM-1), demonstrate adipocyte-specific cell-surface expression (Hong *et al.,* 2005; Song *et al.,* 2009), although very little is known about either protein.

[00107] Systemic delivery approaches to target therapeutic modalities to adipose tissue are thus likely to experience complications due to the lack of total adipocyte-specificity of currently known adipocyte cell surface marker proteins. Known targets are primarily involved in lipid metabolism and thus are often expressed in other tissues involved in other tissues is some way involved in lipid storage or metabolism, including the liver and brown adipose tissue, complicating a systemic delivery approach due to the unknown effect(s) of an miR-155-related

treatment approach in off-target tissues. In addition, several of these adipocyte-selective markers demonstrate nutritional regulation, possibly altering tissue expression levels in response to metabolic dysregulation during obesity and obesity-related disease conditions. Similar to preadipocyte cell-surface marker proteins, however, adipocyte marker proteins may be most useful as a means to facilitate the retention of a therapeutic modality within adipose tissue depots or selectively targeted adipose regions. Finally, mature adipocytes express significantly less miR-155 than preadipocytes and miR-155 expression in adipocytes, unlike preadipocytes, is not altered during obesity, suggesting little therapeutic benefit to targeting miR-155 expression in mature adipocytes.

[00108] An miR-155-based pharmaceutical formulation of the present invention can be delivered using either systemic or region-specific (cell-specific, tissue-specific, organ-specific, *etc.*) delivery approaches. Systemic delivery approaches primarily include venous delivery of an miR-155 composition, and particularly includes those miR-155 molecules that have been structurally and/or chemically modified to improve their effective *in vivo* half-life, and/or improve or enhance target specificity. Expression constructs expressing miR-155 compounds, mimetics, analogs or inhibitors thereof may be prepared using any of several molecular approaches known to those of ordinary skill in the pharmaceutical arts to increase the adipose-, preadipocyte- or adipocyte-specific or -selective delivery and/or transfection efficiency of the therapeutic agent to the cells/tissues/regions/organs of interest. Additional delivery approaches include, but are not limited to, lipid- or aptamer-conjugated nucleic acid or nucleic acid derivatives, viral particle-based delivery, and receptor-, aptamer- or antibody-tagged liposomes, nanoparticles, dendrimers or other nucleic acid delivery systems. In certain embodiments, the miR-155 compounds will preferably be expressed under the control of one or more constitutive, inducible, or cell-specific or cell-selective promoter and/or enhancer sequences that have been operably linked to the miR-155 compounds.

[00109] Systemic delivery approaches of small RNA molecules or RNA-derivatives can require large quantities of the therapeutic agent in order to achieve desired effects in targeted tissues or cell types due to capture, degradation and/or clearance in off-target tissues. Successful systemic delivery approaches are therefore expected to require good perfusion of the target tissue with targeted cell types within the tissue receiving substantial exposure to the therapeutic agent. Adipose tissue is well perfused, however, unlike many other common therapeutic targets, such as liver and heart tissue, there is no direct contact between venous blood and adipocytes and preadipocytes in any therapeutic agent targeting adipocytes,

preadipocytes or any other component of adipose stromal vasculature should pass through the vascular endothelium with relatively high efficiency in order to achieve the degree of interstitial accumulation expected to be required for biological activity on the targeted cell type.

[00110] Delivery of RNA-based therapeutic molecules across the blood brain barrier presents even more severe challenges due to the tighter arrangement of vascular endothelial cells in brain capillaries *vs.* other vascular beds, and suggests worst-case limitations that may affect delivery of RNA-based therapeutics across the adipose vascular endothelium. Recently, two approaches have gained popularity as a means for systemic delivery of RNAi molecules to central nervous system targets: 1) chemically modified RNAi/liposomal complexes coupled to ligands that target the complex to specific receptors on the blood-brain barrier and specific cell targets within the brain and 2) direct coupling of RNAi molecules to ligands recognized by receptors on the blood-brain barrier as well as ligands recognized by the targeted tissue within the brain (Mathupala, 2009). Similar delivery approaches are expected to demonstrate comparable or better success in delivery of small RNA-based therapeutics across the adipose vascular bed. While there are currently no well-defined targets specific for capillary vascular endothelial cells within adipose tissue, screening of vascular endothelial cells derived from different tissues has identified genes that display heterogeneous expression among these tissues (Caprioli *et al.,* 2004), suggesting that cell-surface vascular endothelial cell markers that confer adipose-specificity or -selectivity may exist. Once across the adipose vascular endothelium, secondary ligands could be used to increase the specificity or efficiency of RNA-based agents.

[00111] Currently there are no well-defined cell surface proteins or molecules that demonstrate complete preadipocyte- or adipocyte- specificity, since several such preadipocyte and adipocyte markers demonstrate significant expression in additional tissues. However, combinatorial selection approaches that selectively enrich adipose tissue delivery and preadipocyte or adipocyte specificity could significantly increase the overall specific of a systemic delivery approach. Similarly, the effective dose of an RNA-based therapeutic agent within the adipose tissue interstitium could be increased by increasing its cellular uptake, with or without increasing the cell-specificity of uptake. One possible approach to achieve this is through conjugation of a cholesterol group, or a long-chain fatty acid, to the RNA therapeutic agent, since this approach has been shown to increase siRNA delivery to the liver (Soutschek *et al.,* 2004), another tissue with significant lipid storage and metabolic activity. Finally, the specificity for and therapeutic dose within specific adipose tissue cell types could be further enhanced through the use of recombinant DNA constructs expressing miR-155 mimic or

inhibitor constructs from promoters conferring preadipocyte- or adipocyte-selective expression. For example, fibroblast growth factor 10 is highly expressed in preadipocytes but not other adipose tissue cell types (Yamasaki *et al.,* 1999), while adiponectin is highly expressed only in mature adipocytes.

[00112] Systemic delivery approaches may be appropriate for treatment modalities where it is desirable globally regulate adipocyte or preadipocyte cell function. However, adipose tissue is localized in several depots, several of which are closely associated with major organs, such as heart and kidney, and may therefore directly impact upon their function through direct endocrine actions. Moreover, adipose tissue can be divided into white and brown adipose tissue, which demonstrate significantly different histology and function, but share several preadipocyte and adipocyte markers in common. Finally, recent literature examining the relative function of different adipose tissue depots has revealed that even white adipose tissue from different anatomical sites (*e.g.,* subcutaneous *vs.* visceral) displays differential gene expression, and that differential accumulation of adipose tissue at these sites can have markedly different metabolic effects. Due to these differences, delivery approaches targeted to specific anatomical sites may prove to be the most desirable way to modulate the localization and function of adipose tissue in a whole body context. Site-specific delivery through selective vascular permeabilization at target sites or direct injection within target tissue should also reduce therapeutic doses required for the desired biological effects and thus lessen the likelihood of off-target affects.

[00113] Site-specific delivery of RNA-based therapeutic agents can be achieved through several means, including high-frequency ultrasound mediated localization of systemically administered agents or direct application or injection of a therapeutic to the desired target site. A high-frequency ultrasound (HIFU) approach selectively targets therapeutic delivery to specific anatomical site by using ultrasound treatment to increase the vascular permeability with the target site, and is suitable for targeted delivery to both subcutaneous and visceral adipose depots. Site-specific therapeutic delivery by injection or transdermal delivery is most suitable for targeting subcutaneous adipose depots, although injection-mediated delivery approaches could also be used to target visceral and other deep adipose tissue depots if coupled with a means of visualizing the targeted injection site (*e.g.,* ultrasound).

4.7 VIRAL VECTOR DELIVERY

[00114] In certain aspects of the invention, it may be desirable to introduce the miR155 compositions to one or more cells, tissues, or body regions of a mammal by viral-vectored gene

therapy approaches. Methods for viral-based gene therapies are well known to those of ordinary skill in the art, including for example adenovirus- (AV), herpes simplex virus- (HSV), and adeno-associated virus (AAV)-based delivery regimens.

[00115] Viral vectors such as AAV have been show to be of particular relevance to the treatment of mammalian diseases. AAV is a human parvovirus that can be propagated both as a lytic virus and as a provirus. The viral genome consists of linear single-stranded DNA, 4679 bases long, flanked by inverted terminal repeats of 145 bases. For lytic growth, AAV requires co-infection with a helper virus. Either AV or HSV can supply helper functions. Without helper, there is no evidence of AAV-specific replication or gene expression. When no helper is available, AAV can persist as an integrated provirus.

[00116] AAV is also particularly attractive for transfer of heterologous nucleic acids to selected mammalian recipient cells because it does not induce any pathogenic response in mammals, and can integrate into the host's cellular DNA (Kotin *et al.*, 1990). AAV-based vectors have been shown to drive higher and longer transgene expression than the identical plasmids lacking the TRs of AAV in most cell types.

[00117] Several factors prompted researchers to exploit rAAV as an expression vector for mammalian cells. One is that the requirements for delivering a gene to integrate into the host chromosome are surprisingly few. It is necessary to have the 145-bp ITRs, which are only 6% of the AAV genome. This leaves room in the vector to assemble a 4.5-kb DNA insertion. While this carrying capacity may prevent the AAV from delivering large genes, it is amply suited for delivering the polynucleotide constructs of the present invention.

[00118] AAV vectors (as well as pluralities of viral particles and infectious virions comprising such vector sequences) have also proven to be excellent delivery vehicles in mammalian cells due to their safety, relatively complicated rescue mechanism (not only wild type adenovirus but also AAV genes are required to mobilize rAAV), non-pathogenicity, and lack of association with any known mammalian disease. The removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, recombinant AAV (rAAV) does not evoke an inflammatory response. AAV-based viral vectors therefore, provide many desirable features for delivery of the miR-155 polynucleotide constructs to mammalian cells in the practice of the present invention.

[00119] Recombinant vectors and pluralities of isolated mammalian host cells that include such vectors also form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid

in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, an miR-155 nucleic acid sequence.

[00120] Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

[00121] In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the DNA segment of interest under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an miR-155 sequence in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

[00122] Naturally, it will be important to employ a promoter that effectively directs the expression of an miR-155 nucleic acid segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced nucleic acid segment.

[00123] The particular promoter that is employed to control the expression of the selected nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell(s), tissue(s) or organ(s) of interest. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Such promoters typically include either human- or viral-derived promoters, such as, without limitation, a CMV promoter, an HSV promoter, or one or more mammalian cell-specific promoters.

[00124] In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the selected nucleic acid sequence. The use of

other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a genetic sequence such as one encoding miR-155 is contemplated as well, provided the level of expression is sufficient for the chosen purpose.

4.8 PHARMACEUTICAL FORMULATIONS

[00125] In certain embodiments, the present invention concerns formulation of one or more miR-155 compounds in a pharmaceutically acceptable composition for administration to a cell or an animal, either alone, or in combination with one or more other modalities of diagnosis, prophylaxis and/or therapy. The formulation of pharmaceutically acceptable excipients and carrier solutions is well known to those of ordinary skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens.

[00126] In certain circumstances it will be desirable to deliver the adipogenesis-modulating compositions disclosed herein in suitably-formulated pharmaceutical vehicles by one or more standard delivery devices, including, without limitation, subcutaneously, parenterally, intravenously, intramuscularly, intrathecally, orally, intraperitoneally, transdermally, topically, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs within or about the body of an animal.

[00127] The methods of administration may also include those modalities as described in U.S. Patents 5,543,158; 5,641,515, and 5,399,363, each of which is specifically incorporated herein in its entirety by express reference thereto. Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in sterile water, and may be suitably mixed with one or more surfactants, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, oils, or mixtures thereof. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[00128] For administration of an injectable aqueous solution, without limitation, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, transdermal, subdermal, and/or intraperitoneal administration. In this regard, the compositions of the present invention may be formulated in one or more pharmaceutically acceptable vehicles, including for example sterile aqueous media, buffers, diluents, *etc.* For example, a given dosage of active ingredient(s) may be dissolved in a particular volume of an isotonic solution (*e.g.,* an isotonic NaCl-based solution), and then

injected at the proposed site of administration, or further diluted in a vehicle suitable for intravenous infusion (*see*, *e.g.*, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). While some variation in dosage will necessarily occur depending on the condition of the subject being treated, the extent of the treatment, and the site of administration, the person responsible for administration will nevertheless be able to determine the correct dosing regimens appropriate for the individual subject using ordinary knowledge in the medical and pharmaceutical arts.

[00129] Sterile injectable compositions may be prepared by incorporating the disclosed drug delivery vehicles in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions can be prepared by incorporating the selected sterilized active ingredient(s) into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above. The compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein), and which are formed with inorganic acids such as, without limitation, hydrochloric or phosphoric acids, or organic acids such as, without limitation, acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, without limitation, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation, and in such amount as is effective for the intended application. The formulations are readily administered in a variety of dosage forms such as injectable solutions, topical preparations, oral formulations, including sustain-release capsules, hydrogels, colloids, viscous gels, transdermal reagents, intranasal and inhalation formulations, and the like.

[00130] The amount, dosage regimen, formulation, and administration of the compositions disclosed herein will be within the purview of the ordinary-skilled artisan having benefit of the present teaching. It is likely, however, that the administration of a therapeutically-effective, pharmaceutically-effective, prophylactically-effective, or diagnostically-effective amount of the disclosed pharmaceutical compositions may be achieved by a single administration, such as, without limitation, a single injection of a sufficient quantity of the delivered agent to provide the desired benefit to the patient undergoing such a procedure. Alternatively, in some circumstances, it may be desirable to provide multiple, or successive

administrations of the adipogenesis-modulatory compositions disclosed herein, either over a relatively short, or even a relatively prolonged period, as may be determined by the medical practitioner overseeing the administration of such compositions to the selected individual.

[00131] Typically, formulations of one or more active ingredients in the drug delivery formulations disclosed herein will contain an effective amount for the selected therapy or diagnosis. Preferably, the formulation may contain at least about 0.001% of each active ingredient, preferably at least about 0.01% of the active ingredient, although the percentage of the active ingredient(s) may, of course, be varied, and may conveniently be present in amounts from about 0.01 to about 90 weight % or volume %, or from about 0.1 to about 80 weight % or volume %, or more preferably, from about 0.2 to about 60 weight % or volume %, based upon the total formulation. Naturally, the amount of active compound(s) in each composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological $t_{1/2}$, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one of ordinary skill in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable. Preferably, the compositions of the present invention may be administered in the same dosage amount as a unifunctional or unfunctionalized pharmaceutical compositions while inhibiting or avoiding one or more adverse effects of such unifunctional or unfunctionalized composition.

[00132] The pharmaceutical compositions disclosed herein may be administered by any effective method, including, without limitation, by parenteral, intravenous, intramuscular, or even intraperitoneal administration as described, for example, in U.S. Patents 5,543,158, 5,641,515 and 5,399,363 (each of which is specifically incorporated herein in its entirety by express reference thereto). Solutions of the active compounds as free-base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose, or other similar fashion. The pharmaceutical forms adapted for injectable administration include sterile aqueous solutions or dispersions, and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions including without limitation those described in U.S. Patent 5,466,468 (which is specifically incorporated herein in its entirety by express reference thereto). In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be at least sufficiently stable under the conditions of manufacture and storage, and must be preserved

against the contaminating action of microorganisms, such as viruses, bacteria, fungi, and such like.

[00133] The carrier(s) can be a solvent or dispersion medium including, without limitation, water, ethanol, polyol (*e.g.,* glycerol, propylene glycol, and liquid polyethylene glycol, and the like, or a combination thereof), one or more vegetable oils, or any combination thereof, although additional pharmaceutically-acceptable components may be included.

[00134] Proper fluidity of the pharmaceutical formulations disclosed herein may be maintained, for example, by the use of a coating, such as *e.g.,* a lecithin, by the maintenance of the required particle size in the case of dispersion, by the use of a surfactant, or any combination of these techniques. The inhibition or prevention of the action of microorganisms can be brought about by one or more antibacterial or antifungal agents, for example, without limitation, a paraben, chlorobutanol, phenol, sorbic acid, thimerosal, or the like. In many cases, it will be preferable to include an isotonic agent, for example, without limitation, one or more sugars or sodium chloride, or any combination thereof. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example without limitation, aluminum monostearate, gelatin, or a combination thereof.

[00135] While systemic administration is contemplated to be effective in many embodiments of the invention, it is also contemplated that formulations of the disclosed drug delivery compositions may be suitable for direct injection into one or more organs, tissues, or cell types in the body. Such injection sites include, without limitation, the circulatory system, the spinal cord, the lymphatic system, a joint or joint capsule, a synovium or subsynovium tissue, tendons, ligaments, cartilages, bone, periarticular muscle or an articular space of a mammalian joint, as well as direct administration to an organ or tissue site such as the heart, liver, lung, pancreas, intestine, brain, bladder, kidney, or other site within the patient's body, including, for example, without limitation, introduction of the delivered therapeutic or diagnostic agent(s) *via* intra-abdominal, intra-thoracic, intravascular, or intracerebroventricular delivery of a suitable liposomal formulation. Administration of the disclosed compositions need not be restricted to one or more of these delivery means, but instead may be conducted using suitable means, including those known to the one of ordinary skill in the relevant medical arts. In certain embodiments, the active ingredients of the invention may be formulated for delivery by needle, catheter, and related means, or alternatively, may be included within a medical device, including, without limitation, drug-eluting implants, stents, catheters, and such

like. The formulations may also be prepared for injection by an implanted drug-delivery pump or similar mechanism.

[00136] The administration of the pharmaceutical compositions disclosed herein may be conducted using any method as conventionally employed in the medical arts, and may include, without limitation, administration of intranasal sprays, inhalation, and/or other aerosol delivery vehicles (see *e.g.,* U.S. Patents 5,756,353 and 5,804,212, each of which is specifically incorporated herein in its entirety by express reference thereto). Delivery of drugs using intranasal microparticle resins (see *e.g.,* Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (see *e.g.,* U.S. Patent 5,725,871, specifically incorporated herein in its entirety by express reference thereto) are also well known to those of ordinary skill in the pharmaceutical arts, and may also be employed in the practice of the present methods. Transmucosal drug delivery is also contemplated to be useful in the practice of the invention. Exemplary methods are described, for example, without limitation, in U.S. Patent 5,780,045 (specifically incorporated herein in its entirety by express reference thereto).

[00137] The disclosed pharmaceutical formulations may also be administered through transdermal or other topical administration routes. Exemplary methods for the use of liposomal formulations in topical therapy are found, for example, in U.S. Patents 5,540,936, and 6,133,451 (each of which is specifically incorporated herein in its entirety by express reference thereto).

[00138] In particular embodiments, the disclosed pharmaceutical compositions may be formulated using one or more pharmaceutical buffers, vehicles, or diluents, and intended for administration to a mammal through a suitable route, such as, by intramuscular, intravenous, subcutaneous, intrathecal, intra-abdominal, intravascular, intra-articular, or alternatively, by direct injection to one or more cells, tissues, or organs of such a mammal.

[00139] The pharmaceutical formulations disclosed herein are not in any way limited to use only in humans, or even to primates, or mammals. In certain embodiments, the methods and compositions disclosed herein may be employed using avian, amphibian, reptilian, or other animal species.

[00140] In preferred embodiments, however, the compositions of the present invention are preferably formulated for administration to a mammal, and in particular, to humans, in a variety of diagnostic, therapeutic, and/or prophylactic regimens. The compositions disclosed herein may also be provided in formulations that are acceptable for veterinary administration, including, without limitation, to selected livestock, exotic or domesticated animals, companion

animals (including pets and such like), non-human primates, as well as zoological or otherwise captive specimens, and such like.

[00141] Such methods may also encompass prophylactic treatment of one or more animals suspected of having, or at risk for developing one or more such conditions either following diagnosis, or prior to the onset of symptoms. To that end, in certain embodiments the pharmaceutical compositions disclosed and/or described herein may also find utility in the area of vaccine development, and antigen administration/vaccination and the like.

4.9 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

[00142] In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the compositions of the present invention into suitable host cells. In particular, the compositions of the present invention may be formulated for delivery encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

[00143] Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids disclosed herein. The formation and use of liposomes is generally known to those of ordinary skill in the art (see for example, U. S. Patent 5,741,516, specifically incorporated herein in its entirety by express reference thereto). Further, various methods of liposome and liposome like preparations as potential drug carriers have been described (U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587 (each of which is specifically incorporated herein in its entirety by express reference thereto).

[00144] Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way. Exemplary nanoparticle formulations are set forth in U. S. Patent 5,145,684 (specifically incorporated herein in its entirety by express reference thereto) and the references cited therein.

4.10 ADDITIONAL MODES OF DELIVERY

[00145] In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the nucleic acid compositions disclosed herein to one or more particular target cell(s), tissue(s), organ(s), or to the body of an animal systemically. Sonophoresis (*i.e.,* ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein in its entirety by express reference thereto) as

a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback-controlled delivery (U. S. Patent 5,697,899), each of which is specifically incorporated herein in its entirety by express reference thereto.

4.11 THERAPEUTIC AND DIAGNOSTIC KITS

[00146] The invention also encompasses one or more compositions together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular pharmaceutical formulations, and in the preparation of therapeutic agents for administration to a mammal, and in particularly, to a human, using one or more of the miR-155-derived compositions described herein. In particular, such kits may comprise one or more miR-155compositions in combination with instructions for using the composition in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

[00147] As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include non-human primates, murines, ovines, bovines, equines, lupines, porcines, canines, felines, exotics, and animals under veterinary care. The composition may include partially or significantly purified miR-155 compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

[00148] Therapeutic kits may also be prepared that comprise at least one of the miR-155compositions disclosed herein and instructions for using the composition as a therapeutic agent, and particularly in the treatment of one or more conditions such as, without limitation, obesity, excessive weight gain, fatty tissue increase, liposculpture, fat remodeling, and the like. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed miR-155composition(s) may be placed, and preferably suitably aliquotted. Where a second miR-155composition or an additional therapeutic, prophylactic, or diagnostic reagent is also provided, the kit may also

contain a second distinct container into which this second composition may be placed. Alternatively, the plurality of therapeutic, diagnostic, or prophylactic compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container such as a vial, flask, syringe, bottle, or other suitable single container, device, or kit. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.,* injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.12 METHODS OF NUCLEIC ACID DELIVERY AND DNA TRANSFECTION

[00149] In certain embodiments, it is contemplated that one or more of miR-155 compositions as disclosed herein will be used to transfect one or more suitable animal, and preferably mammalian, host cells or tissues. In addition to the methods disclosed above, several non-viral methods for the transfer of expression constructs into cultured mammalian cells are also contemplated for use in certain *in vitro* embodiments, and under conditions where the use of other delivery vehicles for the introduction of miR-155 compositions is less desirable or less feasible. Such non-vial based methods for introducing nucleic acid compositions to cells include, without limitation, calcium phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, high velocity microprojectile bombardment, and receptor-mediated transfection. Some of these techniques may also be successfully adapted for either *in vivo* and/or *ex situ* use as well.

4.13 EXPRESSION IN ANIMAL CELLS

[00150] The inventors contemplate that a polynucleotide comprising a contiguous nucleic acid sequence that encodes an miR-155 composition in accordance with the present invention may also be utilized to treat one or more cellular, metabolic, or structural defects in a population of transformed host cells. Such pluralities of cells preferably include animal cells, with mammalian cells such as those obtained from a human or non-human primate, murine, canine, bovine, equine, epine, or porcine species being particularly preferred. In particular, the use of such constructs for the treatment and/or amelioration of excess body weight gain, fat accumulation, adiposity, fat production, or one or more conditions arising from overeating or obesity in a human subject suspected of suffering from such a disorder is highly contemplated.

4.14 TRANSGENIC ANIMALS

[00151] It is contemplated that in some instances the genome of a transgenic non-human animal of the present invention will have been altered through the stable introduction of one or more of the miR-155 nucleic acid compositions described herein, either native, synthetically modified, or mutated. As used herein, the term "transgenic animal" is intended to refer to an animal that has incorporated exogenous DNA sequences into its genome. In designing a heterologous gene for expression in animals, sequences that interfere with the efficacy of gene expression, such as polyadenylation signals, polymerase II termination sequences, hairpins, consensus splice sites and the like are preferably eliminated. Current advances in transgenic approaches and techniques have permitted the manipulation of a variety of animal genomes *via* gene addition, gene deletion, or gene modifications. It is contemplated in the present invention that transgenic animals contribute valuable information as models for studying the effects of miR-155 compositions on correcting one or more physiological defects, and for treating one or more obesity disorders in an animal.

4.15 EXEMPLARY DEFINITIONS

[00152] "miRNA" or "miR" in the context of the present invention means a non-coding RNA of between about 18 and 25 nucleotides in length which hybridizes to, and regulates the expression of, a coding RNA. In certain embodiments, a miRNA is the product of cleavage of a pre-miRNA by the enzyme Dicer. Examples of suitable miRNA species are described in the online searchable miRNA database known as miRBase (see *e.g.*, http://microrna.sanger.ac.uk/).

[00153] "Pre-miRNA" or "pre-miR" in the context of the present invention means a non-coding RNA having a hairpin structure, which contains an miRNA. In certain embodiments, pre-miRNA is the product of cleavage of a pri-miR by the double-stranded RNA-specific ribonuclease known as Drosha.

[00154] "Stem-loop sequence" as used in the context of the present invention means an RNA having a hairpin structure and containing a native (*i.e.*, mature or wild-type) miRNA sequence. Pre-miRNA sequences and stem-loop sequences may overlap, and examples of stem-loop sequences are described in the searchable miRNA database, miRBase, noted above.

[00155] The term "monocistronic transcript," as used herein, means an miRNA precursor (i.e., a pre-miRNA) that contains a single miRNA sequence.

[00156] The term "polycistronic transcript," as used herein, means an miRNA precursor that contains two or more miRNA sequences.

[00157] An "antisense compound," as used herein, means a compound having a nucleotide sequence that selectively hybridizes to a target, or "sense" nucleic acid. In certain embodiments, an antisense compound is an oligonucleotide having a nucleotide sequence that is substantially complementary to a target nucleic acid.

[00158] The term "miR antagonist" as used herein, means a compound that interferes with, or inhibits the activity of, one or more miRNAs. In particular embodiments, a miR antagonist comprises an antisense compound targeted to an miRNA. In certain embodiments, a miR antagonist comprises a modified oligonucleotide having a nucleotide sequence that is substantially complementary to the nucleotide sequence of an miRNA, or a precursor thereof. In particular embodiments, a miR antagonist is a miR-155 antagonist. In other embodiments, a miR-155 antagonist comprises a small molecule, or the like that interferes with or inhibits the activity of a miR-155 miRNA. "miR-155 antagonists" are compounds that interfere with, or inhibit the activity of, miR-155.

[00159] As used herein, the term "carrier" is intended to include any solvent(s), dispersion medium, coating(s), diluent(s), buffer(s), isotonic agent(s), solution(s), suspension(s), colloid(s), inert(s) or such like, or a combination thereof, that is pharmaceutically acceptable for administration to the relevant animal. The use of one or more delivery vehicles for chemical compounds in general, and peptides and epitopes in particular, is well known to those of ordinary skill in the pharmaceutical arts. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the diagnostic, prophylactic, and therapeutic compositions is contemplated. One or more supplementary active ingredient(s) may also be incorporated into, or administered in association with, one or more of the disclosed immunogenic compositions.

[00160] As used herein, the term "expression" refers to the biological production of a product encoded by a coding sequence. In most cases, a polynucleotide (*i.e.,* DNA) sequence, including the coding sequence, is transcribed to form a messenger-RNA (mRNA). The messenger-RNA is then translated to form a polypeptide product that has a relevant biological activity. The process of expression may involve further processing steps to the RNA product of transcription, such as splicing to remove introns, and/or post-translational processing of a polypeptide product.

[00161] As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter that does not naturally occur adjacent to the

referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

[00162] As used herein, the term "operably linked" refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences, or at least one of each, in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. "Operably linked" means that the nucleic acid sequences being linked are typically contiguous, or substantially contiguous, and, where necessary to join two protein coding regions, contiguous and in reading frame. Since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths; however, some polynucleotide elements may be operably linked but not contiguous.

[00163] The phrases "isolated" or "biologically pure" refer to material that is substantially, or essentially, free from components that normally accompany the material as it is found in its native state. Thus, isolated peptides in accordance with the invention preferably are at least essentially, or are free, of materials normally associated with the peptides in their *in situ* environment.

[00164] "Link" or "join" refers to any method known in the art for functionally connecting two or more molecules, including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, electrostatic bonding, and such like.

[00165] As used herein, the term "monoclonal," when used in reference to an antibody, refers to an antibody that is based upon, obtained from or derived from a single clone, including any eukaryotic, prokaryotic, or phage clone. The term monoclonal antibody is often abbreviated "MAb" in the singular, and "MAbs" in the plural.

[00166] As used herein, the term "patient" (also interchangeably referred to as "host" or "subject") refers to any host that can receive one or more of the pharmaceutical compositions disclosed herein. Preferably, the subject is a vertebrate animal, which is intended to denote any animal species (and preferably, a mammalian species such as a human being). In certain embodiments, a "patient" refers to any animal host including without limitation any mammalian host. Preferably, the term refers to any mammalian host, the latter including but not limited to, human and non-human primates, bovines, canines, caprines, cavines, corvines,

epines, equines, felines, hircines, lapines, leporines, lupines, murines, ovines, porcines, ranines, racines, vulpines, and the like, including livestock, zoological specimens, exotics, as well as companion animals, pets, and any animal under the care of a veterinary practitioner. A patient can be of any age at which the patient is able to respond to inoculation with the present vaccine by generating an immune response. In particular embodiments, the mammalian patient is preferably human.

[00167] The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that preferably do not produce an allergic or similar untoward reaction when administered to a mammal, and in particular, when administered to a human. As used herein, "pharmaceutically acceptable salt" refers to a salt that preferably retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects. Examples of such salts include, without limitation, acid addition salts formed with inorganic acids (*e.g.,* hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like); and salts formed with organic acids including, without limitation, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic (embonic) acid, alginic acid, naphthoic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalenedisulfonic acids, polygalacturonic acid; salts with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; salts formed with an organic cation formed from *N,N'*-dibenzylethylenediamine or ethylenediamine; and combinations thereof.

[00168] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and includes any chain or chains of two or more amino acids. Thus, as used herein, terms including, but not limited to "peptide," "dipeptide," "tripeptide," "protein," "enzyme," "amino acid chain," and "contiguous amino acid sequence" are all encompassed within the definition of a "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with, any of these terms. The term further includes polypeptides that have undergone one or more post-translational modification(s), including, without limitation, glycosylation, acetylation, phosphorylation, amidation, derivatization, proteolytic cleavage, post-translation processing, or modification by inclusion of one or more non-naturally occurring amino acids. Throughout the disclosure, common one-letter and three-letter amino acid abbreviations have been employed following the conventional nomenclature in the art: Alanine (A; Ala), Arginine (R; Arg), Asparagine (N; Asn), Aspartic Acid (D; Asp), Cysteine (C; Cys), Glutamine (Q; Gln), Glutamic Acid (E; Glu), Glycine (G; Gly), Histidine

(H; His), Isoleucine (I; Ile), Leucine (L; Leu), Methionine (M; Met), Phenylalanine (F; Phe), Proline (P; Pro), Serine (S; Ser), Threonine (T; Thr), Tryptophan (W; Trp), Tyrosine (Y; Tyr), Valine (V; Val), and Lysine (K; Lys). Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid residue provided the desired properties of the polypeptide are retained. All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

[00169] "Protein" is used herein interchangeably with "peptide" and "polypeptide," and includes both peptides and polypeptides produced synthetically, recombinantly, or *in vitro* and peptides and polypeptides expressed *in vivo* after nucleic acid sequences are administered into a host animal or human subject. The term "polypeptide" is preferably intended to refer to any amino acid chain length, including those of short peptides from about 2 to about 20 amino acid residues in length, oligopeptides from about 10 to about 100 amino acid residues in length, and longer polypeptides including from about 100 amino acid residues or more in length. Furthermore, the term is also intended to include enzymes, *i.e.,* functional biomolecules including at least one amino acid polymer. Polypeptides and proteins of the present invention also include polypeptides and proteins that are or have been post translationally modified, and include any sugar or other derivative(s) or conjugate(s) added to the backbone amino acid chain.

[00170] As used herein, the term "substantially free" or "essentially free" in connection with the amount of a component preferably refers to a composition that contains less than about 10 weight percent, preferably less than about 5 weight percent, and more preferably less than about 1 weight percent of a compound. In preferred embodiments, these terms refer to less than about 0.5 weight percent, less than about 0.1 weight percent, or less than about 0.01 weight percent.

[00171] As used herein, the term "substantially homologous" encompasses sequences that are similar to the identified sequences, such that antibodies raised against peptides having the identified sequences will react with peptides having the substantially homologous sequences. In some variations, the amount of detectable antibodies induced by the homologous sequence is identical to the amount of detectable antibodies induced by the identified sequence. In other variations, the amounts of detectable antibodies induced are substantially similar, thereby providing immunogenic properties. For example, "substantially homologous" can refer to at least about 75%, preferably at least about 80%, and more preferably at least about 85% or

at least about 90% identity, and even more preferably at least about 95%, more preferably at least about 97% identical, more preferably at least about 98% identical, more preferably at least about 99% identical, and even more preferably still, at least substantially or entirely 100% identical (*i.e.*, "invariant").

[00172] As used herein, the terms "treatment," "treat," "treated," or "treating" refer to therapy, or to the amelioration or the reduction, in the extent or severity of disease, or a symptom thereof, whether before or after its development afflicts a patient. When used with respect to an infectious disease, for example, the terms refer to a treatment protocol or regimen that decreases the severity of the infection or decreases or lessens or delays one or more symptoms of illness attributable to the infection, as well as increasing the ability of the infected individual to fight the infection, including *e.g.*, the reduction and/or elimination of the infection from the body of the individual, or to lessen or prevent the disease from becoming worse.

[00173] As used herein, the terms "prevention," "prevent," "prevented," or "preventing" refer to prophylaxis, or to the anticipatory avoidance of disease, whether before or after its development afflicts a patient. Prevention can encompass full or partial inhibition of a given condition, and in certain embodiments concerns the complete prevention of the condition either for a discreet period of time, or for substantially all, or the entirety of the life of the animal receiving the prophylactic administration of one of more compounds of the present invention.

[00174] The term "for example" or "*e.g.*," as used herein, is used merely by way of example, without limitation intended, and should not be construed as referring only those items explicitly enumerated in the specification.

[00175] In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denote "one or more."

5. EXAMPLES

[00176] The following examples are included to demonstrate illustrative embodiments of the invention. It should be appreciated by those of ordinary skill in the art that the techniques disclosed in the examples that follows represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of ordinary skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 -- SEVERAL MIRNAS ARE DIFFERENTIALLY EXPRESSED DURING ADIPOGENIC DIFFERENTIATION OF 3T3-L1 CELLS

[00177] This example describes the identification of miRNAs that regulate adipogenesis using miRNA microarrays to identify miRNAs whose expression were altered during the differentiation of 3T3-L1 preadipocytes to mature adipocytes.

5.1.1 EXPERIMENTAL PROCEDURES

[00178] **Cell Culture Conditions:** Replica plates of 3T3-L1 preadipocytes were grown to confluence in DMEM (Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). At day 0, confluent 3T3-L1 cell cultures were then switched to differentiation medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX and 10 μg/mL insulin) to induce adipocyte differentiation. Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were commercially available from Sigma Chemical Co. (St. Louis, MO, USA). After 2 days culture in differentiation medium, the cells were switched to adipocyte culture medium (DMEM supplemented with 10% FBS and 10 μg/mL insulin) for the remainder of the culture period. Replica plates were harvested for RNA isolation 0, 1, 3, and 8 days after induction of adipogenesis. *In vitro* adipogenesis in 3T3-L1 cultures was detected by Oil Red O staining as described elsewhere (Soukas *et al.*, 2001).

[00179] **miRNA Array Analyses:** Replica plates were harvested 0, 1, 3, and 8 days after induction of adipogenesis and total 3T3-L1 cell RNA was isolated with TRIzol Reagent (Invitrogen). Low-molecular-weight (LMW) RNA was isolated the from total RNA using mirVana RNA Isolation Kit (Ambion, Austin, TX, USA), and then 4 μg of each LMW sample was labeled with 500 ng of 5'-phosphate-cytidyl-uridyl-cy3-3' (Thermo Scientific Dharmacon, Lafayette, CO, USA) using 2 unit of T4 ligase (New England Biolabs). Labeled RNA samples were precipitated with 70% ethanol, and hybridized with miRNA array slides fabricated by CapitalBio Corp. (Beijing, China) according to the manufacturer's instructions (Guo *et al.*, 2008). After miRNA hybridization, slides were washed by two consecutive 5 min 42°C incubations in washing solution (2X SSC, 0.2% SDS) followed by a 5-min room temperature incubation in 0.2 X SSC, and then scanned for hybridized RNA fluorescent signal using a LuxScan 10K-A laser confocal scanner (CapitalBio Corp.). Regulated miRNAs were defined as those whose expression changed more than two-fold at any time point during 3T3-L1 adipogenesis and all identified miRNAs were analyzed by hierarchical cluster analysis using

Cluster 3.0 software (Van Huissteden, 1990) to identify miRNAs with similar expression patterns during adipocyte differentiation.

[00180] **RT-PCR Analyses:** RNA samples isolated from 3T3-L1 cells 0, 1, 3, and 8 days after induction of adipogenesis were analyzed by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to confirm results obtained by miRNA analysis. Total 3T3-L1 cell or tissue RNA used in these studies was isolated with miRNeasy Mini Kits (Qiagen), and reverse-transcribed to cDNA using either TaqMan® Reverse Transcription Reagents (Life Technologies Corp.) for gene detection or a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.) for miRNA detection. Quantitative RT-PCR assays were performed using a 7900HT Fast Real-Time PCR system (Life Technologies Corp.) according to manufacturer's instructions. Gene and miRNA expression levels were normalized against the expression of the 'housekeeping genes' 18S and U6 snRNA expression, respectively, which were analyzed on the same plates. TaqMan® primer and probe sets for miR-155, miR-218, miR-221, miR-222, FABP4, C/EBPα and PPARγ were commercially available from Life Technologies Corp.

[00181] **Plasmid constructs and retroviral infection:** The retrovirus production plasmid, pBABE-puro, and the retrovirus packaging plasmid, pCL-Eco, were commercially available from Addgene (Cambridge, MA, USA). An ~400-bp region of mouse genomic DNA containing the full-length miR-155 was PCR amplified using a sense strand (5′-CGGGATCCCTTGCCTTCAGATTCATAATCGTT-3′) (SEQ ID NO:6) containing a *Bam*HI site and an anti-sense strand (5′-CGGAATTCTGGGCTTGAAGTTGAGATGTTGT-3′) (SEQ ID NO:7) primer containing an *Eco*RI site. This miR-155 DNA locus fragment was then subcloned into the *Bam*HI-*Eco*RI sites of the pBABE-puro plasmid to generate a miR-155 expression plasmid (pBABE-puro-miR155), where miR-155 expression was driven by the 5′ LTR of the pBABE plasmid. Retroviruses were produced by co-transfecting HEK293T cells cultured in DMEM and 5% FBS with pBABE-puro or pBABE-puro-miR155 and pCL-Eco packing plasmid using FuGENE® 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA). Retroviral supernatants collected 2 days after infection were supplemented with 8 μg/mL polybrene (Sigma Chemical Co.) and added to confluent 3T3-L1 cell cultures for 2 days to permit infection. After 2 days exposure to retrovirus, 3T3-L1 cell cultures were supplemented with 2 μg/mL puromycin (InvivoGen, San Diego, CA, USA) and cultured for 14 days to select for infected cells with stable retroviral integration. After 14 days of puromycin selection, surviving 3T3-L1 cells infected with pBABE-puro or pBABE-puro-

miR155 were considered to contain stable retroviral integrants and were used for miR-155 overexpression studies. Retroviral constructs and 3T3-L1 cells carrying retroviral integrants for miR-218, -221 and -222 were generated by the same procedure. Briefly, miR-218, -221 and -222 genomic DNA regions were amplified using sense and anti-sense primer pairs [miR-218 sense (5′-GGGGTACCGTGTTTCCAGGCACCTTCGG-3′) (SEQ ID NO:8) and anti-sense (5′-CCGGATATCGGGCTTTTCAAGGATAATCGATA-3′) (SEQ ID NO:9) primers; miR-221 sense (5′-GGGGTACCATCATGTCCCTATCTGTACTTCCA-3′) (SEQ ID NO:10) and anti-sense (5′-CCGGATATCAATCACAGTGAGACCATTATTTGC-3′) (SEQ ID NO:11) primers; and miR-222 sense (5′-GAAGATCTGGAAGTGAATCTAAAGGTAGTTAAGG-3′) (SEQ ID NO:12) and anti-sense (5′-CCGCTCGAGGGGGAAAGAGGAAGACAA GAAGTA-3′) (SEQ ID NO:13) primers. PCR-amplified DNA fragments containing miR-218, -221 and -222 were directionally cloned into the *Bam*HI and *Eco*RI sites of the pBABE-puro polylinker using the *KpnI/Eco*RV restriction sites in the sense and anti-sense primers of miR-218 and -221 and the *BglII/Xho*I restriction sites in the sense and anti-sense primers of miR-222.

[00182] Inhibition of 3T3-L1 Adipogenesis by of miR Overexpression: 3T3-L1 preadipocyte cell lines overexpressing miR-155, -218, -221, or -222 from stably integrated pBABE-puro-miR-155, -218, -221, or -222 retrovirus were differentiated using 3T3-L1 differentiation conditions described above. At day 8 post-induction, the relative effect of miR-155, -218, -221, or -222 overexpression on adipocyte differentiation was measured by Oil Red O staining to determine lipid accumulation, RT-PCR to measure mRNA expression of adipocyte marker genes, and Western blot to measure protein expression of adipocyte marker proteins.

[00183] Western Blot Analyses: Whole-cell protein preparation and western blotting were performed as previously described (Deng *et al.,* 2006) using equal amounts of total loaded protein (20-60 μg) per sample. Primary antibodies specific for mouse C/EBPα, C/EBPβ, and PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as was a horseradish peroxidase (HRP)-conjugated anti-actin antibody. Primary antibodies specific for mouse FABP4 was commercially available from R&D Systems. All secondary antibodies were commercially available from Cell Signaling Technology (Danvers, MA, USA).

5.1.2 RESULTS

[00184] A total of forty-two miRNAs were detected that exhibited a two-fold or greater expression change during adipogenesis. Sixteen of these were up-regulated and 26 were down-regulated. Hierarchical clustering of these miRNAs (FIG. 1) identified five distinct groups, the largest of which contained 18 miRNAs whose expression progressively decreased during adipogenesis. In order to validate these results, fourteen miRNAs selected from the five hierarchical clusters were reanalyzed at each time point by quantitative RT-PCR. Comparison of miRNA array and quantitative PCR data revealed a strong correlation (FIG. 2; $r = 0.88$, $p < 0.0001$).

[00185] It was discovered that one of the most down-regulated miRNAs, miR155, was expressed in adipose tissue *in vivo* and was expressed in stromal-vascular fraction nearly two-fold more than in the mature adipocytes. Overexpression of miR-155 in 3T3-L1 cells decreased lipid accumulation and repressed adipocyte marker genes, while antisense-mediated miR-155 inhibition had the opposite effect. Consistent with *in vitro* results, miR-155 knockout mice demonstrated induction in adipose mass versus wild-type mice.

[00186] Since the majority of miRNA identified in the screening study were repressed during adipogenesis, the potential regulatory effect of constitutive overexpression of four of the most down-regulated miRNAs (miR-155, miR-218, miR-221 and miR-222), which were expressed at <15% of the level detected in preadipocytes, was examined. All four miRNAs were robustly overexpressed (> four-fold) in 3T3-L1 preadipocytes after retroviral infection (FIG. 3A). However, only miR-155 demonstrated a reproducible effect to inhibit 3T3-L1 preadipocyte differentiation to mature adipocytes (FIG. 3B). Constitutive overexpression of miR-155 in 3T3-L1 preadipocytes strongly repressed adipocyte differentiation, dramatically attenuating lipid accumulation (FIG. 4A) and markedly reduced adipocyte differentiation markers, including FABP4, C/EBPα and PPARγ mRNA and protein expression (FIG. 4B and FIG. 4C). Conversely, transfection of 3T3-L1 preadipocytes with an miR-155 inhibitor oligonucleotide during adipocyte differentiation markedly enhanced lipid accumulation and significantly increased the mRNA expression of FABP4 and PPARγ (FIG. 5A and FIG. 5B), strongly suggesting that attenuation of adipogenesis by miR-155 was not due to a nonspecific inhibitory mechanism.

[00187] In an initial screen, miR-155 expression in differentiating adipocytes was not markedly different at day 1 and day 3 after induction, but decreased to 10-20% of the initial expression level by day 8 post-induction. Subsequent analyses used to refine the expression

pattern of miR-155 during adipocyte differentiation, revealed that miR-155 expression increased 1.5-fold *vs.* that of preadipocytes (0 hr baseline) by 1-day post-induction, further increased to 2.5-fold baseline by day 2, sharply decreased (0.9-fold baseline) at day 3, and then progressively decreased to 0.2-fold baseline at day 9 post-induction (FIG. 6). Based on the expression pattern of miR-155 during 3T3-L1 adipogenesis and the potent inhibition of adipogenesis by constitutive miR-155 expression, it was concluded that miR-155 expression attenuated the expression of an adipogenic regulatory gene (or genes) required for the late stage of adipogenic differentiation.

[00188] Constitutive miR-155 expression strongly inhibited differentiation of 3T3-L1 preadipocytes into mature adipocytes. However, while 3T3-L1 cells are a well-established *in vitro* culture model for adipocyte differentiation, these cells do not spontaneously differentiate into mature adipocytes and the conditions used to induce adipocyte differentiation are not physiologically relevant. Therefore, the ability of constitutive miR-155 overexpression to inhibit adipocyte differentiation was examined in 3T3-442A preadipocytes, which can spontaneously differentiate to mature adipocytes when implanted into mice and which can be induced to differentiate during *in vitro* culture by treatment with insulin. Constitutive miR-155 overexpression markedly attenuated insulin-induced 3T3-442A lipid accumulation (FIG. 7A) and expression of adipocyte differentiation markers (FIG.7B), including FABP4 and PPARγ, to a similar degree as observed in differentiating 3T3-L1 preadipocytes upon constitutive miR-155 expression. Thus, elevated miR-155 expression appears to inhibit adipogenesis *in vitro*.

5.2 EXAMPLE 2 -- ADIPOSE TISSUE AND PRIMARY ADIPOCYTES EXPRESS MIR-155

[00189] Several reports have demonstrated that miR-155 is differentially expressed during hematopoietic differentiation and induced in vascular tissues in response to inflammatory stimuli. However, miR-155 expression has not been demonstrated in adipocytes or their precursors. In order to confirm that miR-155 is expressed in adipose tissue, the present example details a study in which miR-155 expression was surveyed in a panel of tissues isolated from adult C57BL6/J mice.

5.2.1 EXPERIMENTAL PROCEDURES

[00190] **Tissue Distribution of miR-155 Expression:** Male C57BL/6J mice were obtained from the Jackson Laboratory at 7 weeks of age and fed a standard chow diet (Harlan-Teklad 2920; Harlan-Teklad, Inc., Madison, WI, USA) until sacrificed at 9 weeks-of-age by CO_2 inhalation. Immediately after sacrifice mice were placed on ice and major tissues and

organs were dissected out and flash-frozen in liquid nitrogen for later processing for total RNA isolation. Samples were obtained from white adipose tissue (WAT), brown adipose tissue (BAT), heart, white blood cells (WBC; peripheral blood mononuclear cells), brain, bone marrow (BM), intestine, kidney, liver, lung, skeletal muscle (gastrocnemius), pancreas, spleen, stomach, and aorta (vessel). WBC samples were obtained by RBC lysis of whole blood samples, where the resulting WBC pellets were PBS-washed prior to flash freezing for subsequent RNA isolation. Sample of intestine and stomach were repeatedly flushed with ice-cold PBS before flash freezing in liquid nitrogen. Total RNA was isolated from organ and tissue samples with TRIzol® Reagent (Invitrogen/Life Technologies Corp.), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Invitrogen/Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Invitrogen/Life Technologies Corp.). Tissue miR-155 expression levels were normalized against the expression of the U6 snRNA 'housekeeping RNA', which was analyzed on the same plates. The TaqMan® primer/probe sets for miR-155 analysis were also commercially available from Invitrogen/Life Technologies Corp.

[00191] **miR-155 Expression in Mouse WAT Fractions:** Male C57BL/6J mice were obtained from the Jackson Laboratory at 7 weeks of age and fed a standard chow diet (Harlan-Teklad 2920) until sacrificed at 3-months-of-age by CO_2 inhalation. Adipocytes and stromovascular fraction (SVF: preadipocytes, endothelial cells, vascular smooth muscle cells, macrophages, *etc.*) cells were isolated from the epididymal fat pads as previously described (Halleux *et al.*, 1999).

5.2.2 RESULTS

[00192] As depicted in FIG. 8A, miR-155 expression was detectable at varying degrees in all surveyed tissues, including adipose tissue, with the highest levels observed in white blood cells (WBC), spleen, heart and skeletal muscle (SkM), and lowest levels found in brain, liver, and aorta. White and brown adipose tissue miR-155 expression (WAT and BAT, respectively) was lower than that observed in WBC, spleen, heart and SkM expression, but comparable to expression observed in bone marrow, intestine, kidney, lung and pancreas. Moreover, miR-155 was expressed in both the mature adipocyte and stromal-vascular fraction (SVF) of fractionated mouse epididymal white adipose tissue (FIG. 8B).

5.3 EXAMPLE 3 -- MIR-155 DIRECTLY REGULATES C/EBPβ EXPRESSION

[00193] This example describes the identification and experimental validation of a evolutionarily-conserved miR-155 target site in the 3'-UTR of C/EBPβ, a gene known to play an important role in adipocyte differentiation.

5.3.1 EXPERIMENTAL PROCEDURES

[00194] **Database Searches for miR-155 Target Genes:** Candidates for post-transcriptional regulation by miR-155 were identified by searching the TargetScan and miRBase mRNA target databases to identify genes with conserved regions of complementarity to miR-155 sequence.

[00195] **Cell Culture Conditions:** Replica plates of 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% FBS. Replica plates of HEK293T cells were cultured in DMEM supplemented with 5% FBS. Confluent 3T3-L1 cell cultures were switched to differentiation medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μg/mL insulin) to induce adipocyte differentiation, and replica plates were harvested for RNA and protein isolation 0, 2, 4, 10 and 24 hours after induction. Insulin, dexamethasone, and IBMX were commercially available from Sigma Chemical Co.

[00196] **Western Blot Analyses:** Whole-cell protein preparation and western blotting were performed as previously described (Deng *et al.*, 2006) using equal amounts of total loaded protein (20-60 μg) per sample. Primary antibodies specific for mouse C/EBPα and C/EBPβ, and a horseradish peroxidase (HRP)-conjugated anti-actin antibody were commercially available from Santa Cruz Biotechnology. Secondary antibodies were commercially available from Cell Signaling Technology.

[00197] **RT-PCR Analyses:** Total 3T3-L1 RNA was isolated with miRNeasy Mini Kits (Qiagen), and reverse-transcribed to cDNA using either TaqMan® reverse transcription reagents (Life Technologies Corp.) for gene detection or TaqMan® miRNA Reverse Transcription Kits (Life Technologies Corp.) for miRNA detection. RT-PCR assays were performed using a 7900HT Fast Real-Time PCR system (Life Technologies Corp.). Resulting mRNA and miRNA cDNA samples were RT-PCR analyzed using a 7900HT Fast Real-Time PCR system (Life Technologies Corp.) with mRNA- and miRNA-specific TaqMan® primers and probes (Life Technologies Corp.). RT-PCR expression data for a given mRNA or miRNA

within a sample was normalized against 18S rRNA or U6 snRNA expression within the same sample, measured on the same reaction plate, as a reference control.

[00198] 3T3-L1 Transfection with Synthetic miR-155 Oligonucleotides: Confluent 12-well replica plates of 3T3-L1 preadipocytes were transfected for 6 hours with 40 pmols of a synthetic miR-155 inhibitor or negative control (NC) oligonucelotide (Dharmacon) or a synthetic miR-155 mimic or NC oligonucleotide (Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were harvested to isolate RNA for RT-PCR and Western blot analyses of C/EBPα and C/EBPβ mRNA and protein expression.

[00199] Cloning of mouse C/EBPα 3'-UTR and C/EBPβ 3'-UTR sequence: A 3T3-L1 adipocyte cDNA library was generated from total RNA isolated from differentiated 3T3-L1 adipocytes using a SuperScript Full-Length cDNA Library Construction Kit (Invitrogen). C/EBPα and C/EBPβ mRNA 3'UTR fragments were PCR-amplified from a 3T3-L1 adipocyte cDNA library (Invitrogen). A 491-bp region of C/EBPβ cDNA (32-522 nucleotides 3′ of the mouse C/EBPβ stop codon) containing a predicted miR-155 recognition sequence was PCR-amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs (Ipswich, MA, USA), a sense-strand (5′-cgagctcTGGCCTCGGCGGGCC ACTGCTA-3′) (SEQ ID NO:14) primer containing a *Sac*I restriction site and an anti-sense strand (5′-cccaagcttCTTCACTTTAATGCTCGAAACGGA-3′) (SEQ ID NO:37) primer containing a *Hind*III site. A 1366-bp region of C/EBPα cDNA (107-1472 nucleotides 3′ of the mouse C/EBPα stop codon) was PCR-amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs), a sense-strand (5′-cctactagtGGTCAAGGCCATGGGCAACT-3′) (SEQ ID NO:38) primer containing an *Spe*I site and an anti-sense strand (5′-cgagctcAAGCGGTCCAGCCCTGCTC-3′) (SEQ ID NO:39) primer containing a *Sac*I site. Amplified C/EBPα and C/EBPβ 3'UTR cDNA fragments were then directionally subcloned into the *Sac*I and *Hind*III sites or *Spe*I and *Sac*I sites of pMIR-REPORT vector (Ambion; Austin, TX, USA), respectively, downstream of a firefly luciferase expression cassette, in order to create expression vectors expressing firefly luciferase transcripts containing C/EBPα and C/EBPβ 3'UTR mRNA sequence. A pMIR-REPORT C/EBPβ 3′UTR construct containing a mutated miR-155 recognition site was generated by point mutation of the C/EBPβ 3′UTR miR-155 target site seed sequence (50-53 nucleotides 3′ of the C/EBPβ stop codon: GCAT →

CGTA) with a QuikChange® Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions.

[00200] **Luciferase Reporter Assays:** For Luciferase-C/EBPβ-3'UTR studies, a mixture of 50 ng of renilla control plasmid (pRL-TK, Promega), 350 ng of luciferase reporter plasmid containing a C/EBPβ 3'-UTR with a wild-type or mutant variant of the putative miR-155 recognition sequence, 400 ng of pUC18 plasmid carrier DNA, and 60 pmol of a miR-155 mimic, a miR-27b mimic or a negative control (NC) oligonucleotide (Dharmacon) was diluted in 50 μL of Optimem (Invitrogen) and complexed with 1 μL of Lipofectamine 2000 in 50 μL of Optimem for 20 min to permit liposome formation. For Luciferase-C/EBPα-3'UTR studies, a mixture of 50 ng of renilla control plasmid (pRL-TK, Promega), 350 ng of luciferase reporter plasmid containing a C/EBPα 3'-UTR, and 60 pmol of a miR-155 mimic or negative control (NC) oligonucleotide (Dharmacon), or a C/EBPα-specific silencer RNA (Qiagen) was diluted in 50 μL of Optimem (Invitrogen) and complexed with 1 μL of Lipofectamine 2000 in 50 μL of Optimem for 20 min to permit liposome formation. After 20 min, lipid:DNA complexes were added to 24-well plate replica wells containing 5×10^4 HEK293T cells cultured in DMEM supplemented with 5% FBS. After 24 hours, luciferase activity in these transfected cells was measured using the Dual-Luciferase Reporter Assay System Kit (Promega), according to the manufacturer's instructions. Renilla luciferase activity was used to normalize the Firefly luciferase activity in order to compensate for variation in transfection efficiency among samples. Luciferase activity of the Luciferase-C/EBPβ-3'UTR wild-type and mutant constructs were normalized against the respective construct's activity in HEK293T cells transfected with the negative control oligonucleotide.

5.3.2 RESULTS

[00201] Candidates for post-transcriptional regulation by miR-155 were identified by searching the TargetScan and miRBase mRNA target databases to identify genes with conserved regions of complementarity to miR-155 sequence. CCAAT/enhancer binding protein beta (C/EBPβ), a transcription factor essential for adipocyte differentiation, was identified as a predicted miR-155 target gene by both databases, and the predicted C/EBPβ 3'-UTR miR-155 binding site was conserved across vertebrate species (FIG. 9). 3T3-L1 cells overexpressing miR-155 from an integrated retrovirus construct (pBABE-puro-155) revealed significantly reduced C/EBPβ mRNA and protein expression *vs.* 3T3-L1 control cells (pBABE-puro) that do not overexpress miR-155, both pre- and post-induction with adipocyte

differentiation medium (FIG. 10A and FIG. 10B). C/EBPβ mRNA expression in these cells was significantly reduced at baseline and 10 hours post-induction (FIG. 10A), while C/EBPβ protein expression was significantly reduced at 0, 12 and 24 hours post-induction (FIG. 10B), suggesting that miR-155 expression may attenuate C/EBPβ expression at baseline and early in preadipocyte differentiation. Reductions in C/EBPβ mRNA expression were marked in uninduced preadipocytes and more modest after adipocyte differentiation, roughly paralleling the expression found in control cells, while C/EBPβ protein expression remained markedly reduced throughout early adipogenesis, suggesting that, while miR-155 may regulate both C/EBPβ mRNA and protein expression, a post-translational rather than post-transcriptional effect is most likely to mediate the majority of a proposed miR-155 direct affect on C/EBPβ protein expression.

[00202] Since long-term constitutive miR-155 overexpression, retroviral integration site(s) or retroviral protein expression could potentially alter the phenotype of 3T3-L1 preadipocytes in a non-specific manner, similar experiments were performed in wild-type 3T3-L1 preadipocytes, using synthetic miR-155 mimic and inhibitor oligonucleotides to modulate miR-155 activity. Transfection of 3T3-L1 preadipocytes with miR-155 mimic oligonucleotide significantly reduced C/EBPβ mRNA and protein expression (FIG. 11A and FIG. 11B), while transfection with an miR-155 inhibitor or non-specific control oligonucleotide had no effect. The mRNA expression of C/EBPα, a downstream target of C/EBPβ that does not have a predicted miR-155 recognition site, decreased in cells transfected with the miR-155 mimic, and also trended to increase in cells transfected with the miR-155 inhibitor. Since miR-155 is expressed in preadipocytes, this result suggested that miR-155 inhibitor transfection was either insufficient to alter basal preadipocyte miR-155 activity on C/EBPβ mRNA, or that the miR-155 affect on C/EBPβ mRNA expression was indirect (see Example 5.4). Western blot analysis revealed that, the unlike C/EBPβ mRNA results, C/EBPβ protein expression markedly decreased in 3T3-L1 preadipocytes transfected with the miR-155 mimic and modestly increased in cells transfected with the miR-155 inhibitor (FIG. 11B), suggesting that miR-155 effects on C/EBPβ mRNA expression were mediated primarily by regulation of C/EBPβ protein translation. 3T3-L1 preadipocyte C/EBPα protein expression was marked attenuated in cells transfected with miR-155 mimic and not significantly increased in cells transfected with miR-155 inhibitor, consistent with miR-155 mimic and inhibitor effects on C/EBPα mRNA expression.

[00203] In order to determine if miR-155 directly targets C/EBPβ mRNA, reporter assays were performed in the HEK293T cell line (which has very low endogenous miR-155 levels), using luciferase-C/EBPβ 3′-UTR fusion constructs to assay the effect of the C/EBPβ 3′-UTR miR-155 target site on luciferase activity in cells transfected with an miR-155 mimic or a non-specific oligonucleotide control. Mutation of the miR-155 target site in a Luciferase-C/EBPβ 3′-UTR expression construct completely abolished the ability of a miR-155 mimic to inhibit the expression of luciferase (FIG. 11C), while mutation of this site completely abolished the ability of the transfected miR-155 mimic to suppress luciferase activity. Thus, attenuation of C/EBPβ mRNA and protein expression appeared to result from a direct interaction of miR-155 with the conserved miR-155 target site in the C/EBPβ 3′-UTR, indicating that miR-155 can directly regulate C/EBPβ mRNA and protein expression during adipocyte differentiation. Luciferase activity from a luciferase-C/EBPα-3′-UTR expression construct was not inhibited by cotransfection with a miR-155 mimic oligonucleotide, but was efficiently repressed by a C/EBPα-specific silencer RNA, indicating that miR-155 does not have a direct affect to attenuate C/EBPα mRNA or protein expression through a cryptic miR-155 binding site in the C/EBPα 3′-UTR sequence.

5.4 EXAMPLE 4 -- MIR-155 PARTIALLY EXERTS ITS ANTIADIPOGENIC EFFECT BY DECREASING C/EBPβ EXPRESSION

[00204] This example describes a study that examined the effect of miR-155 overexpression on 3T3-L1 adipogenesis in cells overexpressing C/EBPα and C/EBPβ from retroviral expression constructs lacking C/EBPβ and C/EBPα3′-UTR sequence.

5.4.1 EXPERIMENTAL PROCEDURES

[00205] **Cell Culture Conditions:** Replica plates of 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% FBS. Replica plates of HEK293T cells were cultured in DMEM supplemented with 5% FBS. Confluent 3T3-L1 cell cultures were switched to differentiation medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μg/mL insulin) to induce adipocyte differentiation, and replica plates were Oil red O stained to detect lipid accumulation or harvested for protein isolation. Insulin, dexamethasone, and IBMX were commercially available from Sigma Chemical Co.

[00206] **Plasmid constructs and retroviral infection:** Retrovirus production and packaging plasmids pBABE-hygro, and pCL-Eco, were commercially available from Addgene (Cambridge, MA, USA). A C/EBPβ retrovirus expression construct, pBABE-hygro-LAP2, which does not contain the C/EBPβ 3′-UTR sequence, was commercially available from Addgene. A pBABE-hygro-C/EBPα retroviral plasmid expression construct, lacking C/EBPα 3′-UTR sequence, was created by subcloning C/EBPα cDNA sequence from pSV(X)- C/EBPα (Addgene) into the pBABE-hygro polylinker *Bam*HI site. Retroviruses were produced by co-transfecting HEK293T cells cultured in DMEM and 5% FBS with pBABE-hygro, pBABE-hygro-C/EBPα, or pBABE-hygro-LAP2 (C/EBPβ) and pCL-Eco packing plasmid using Fugene 6 Transfection Reagent (Roche). Retroviral supernatants were collected 2 days after infection were supplemented with 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). 3T3-L1 preadipocytes were co-infected with puromycin-selectable control (pBABE-puro) or miR-155 overexpressing (pBABE-puro-miR155) retrovirus and hygromycin-selectable control (pBABE-hygro), C/EBPα-overexpressing (pBABE-hygro-C/EBPα) or C/EBPβ-overexpressing (pBABE-hygro-LAP2) retrovirus for 2 days, then induced to differentiate by 2 days culture in 3T3-L1 adipocyte differentiation buffer supplemented with 2 μg/mL puromycin (InvivoGen) and 400 μg/mL hygromycin (InvivoGen), then switched to adipocyte culture medium supplemented with supplemented with 2 μg/mL puromycin and 400 μg/mL hygromycin. Replica plates of infected cells were allowed to differentiate for 6 days post-induction and then stained with Oil Red O to detect lipid accumulation as a marker of adipocyte differentiation. Replica plates were harvested to isolate total cellular protein for Western blot analyses at 0, 6, 12, 24, and 48 hours post-induction.

[00207] **Western Blot Analyses:** Whole-cell protein preparation and western blotting were performed as previously described (Deng *et al.*, 2006) using equal amounts of total loaded protein (20-60 μg) per sample. Primary antibody specific for mouse C/EBPα and a horseradish peroxidase (HRP)-conjugated antibody specific for mouse β-actin were commercially available from Santa Cruz Biotechnology. Secondary antibody was commercially available from Cell Signaling Technology.

5.4.2 RESULTS

[00208] C/EBPβ overexpression increased adipogenesis in cells not overexpressing mir-155 (FIG. 12A, top left *vs.* middle left), and partially rescued the adipogenic potential of miR-155-overexpressing cells (FIG. 12A top right *vs.* middle right). However, C/EBPβ stimulation

of adipogenesis was still attenuated by mir-155 overexpression (middle right *vs.* middle left), despite the lack of the C/EBPβ 3′-UTR in the overexpression construct, indicating that mir-155 inhibition of endogenous C/EBPβ expression or the expression of some other gene(s) was sufficient to partially attenuate adipogenesis. Overexpression of C/EBPα, a downstream target of C/EBPβ, induced 3T3-L1 adipogenesis more effectively than C/EBPβ overexpression (FIG. 12A bottom left *vs.* middle left) and C/EBPα enhanced adipogenesis was not attenuated by miR155 overexpression (FIG. 12A bottom left *vs.* bottom right), suggesting that miR-155-mediated effects to attenuate adipogenesis in the face of C/EBPβ overexpression lie upstream of C/EBPα activity.

[00209] C/EBPβ overexpression had no effect to induce C/EBPα protein expression in pBABE-puro control cells (FIG. 12B: top *vs.* middle panel), either at baseline or at any time post-induction, nor did it prevent miR-155-induced attenuation of C/EBPα protein expression (FIG. 12B: top *vs.* middle panel), further indicating that C/EBPβ overexpression was unable to prevent miR-155-mediated repression of its downstream targets.

5.5 EXAMPLE 5 -- MIR-155 EXPRESSION WAS ELEVATED IN ADIPOSE TISSUES IN THREE MURINE MODELS OF OBESITY

[00210] This example describes a study that examined white adipose tissue miR-155 expression in three mouse models of obesity: hyperphagic agouti (A^y) and leptin-receptor-deficient db/db male mice and C57BL/6J male mice with diet-induced obesity.

5.5.1 EXPERIMENTAL PROCEDURES

[00211] **Mouse Obesity Models:** Agouti (A^y) mice and their wild-type controls, db/m, db/db, and low-density lipoprotein receptor deficient ($LDLR^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Agouti (A^y), wild-type, db/m and db/db male mice were fed standard chow diet (Harlan-Teklad 2920) until sacrificed for tissue collection. Wild-type and Agouti (A^y) mice were sacrificed at 6-months-of-age, while db/m and db/db male mice were sacrificed at 3-month-of-age. Male low-density lipoprotein receptor deficient ($LDLR^{-/-}$) mice were fed standard chow diet (Harlan-Teklad 2920) or a high-fat Western diet (RD 12079B; Research Diets, Inc., New Brunswick, NJ, USA) for 12 weeks, starting at 10 weeks-of-age. Mice were sacrificed by CO_2 inhalation and, immediately after sacrifice, and their epididymal fat depots were isolated and either placed immediately on ice prior to tissue sub-fractionation, essentially as previously described (Halleux *et al.,* 1999), or snap-frozen in

liquid nitrogen and stored at -80°C until processed for whole-tissue RNA isolation. Agouti mice and their wild-type controls were maintained in the laboratory of Dr. Qiang Tong (Baylor College of Medicine, Houston, TX, USA) who supplied the epididymal white adipose tissue samples of the 6-month-old Agouti (A^y) and wild-type mice used for analysis.

[00212] **RT-PCR Analyses:** Total RNA was isolated from whole adipose tissue, and adipose tissue adipocyte, preadipocyte and stromovascular fractions using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies Corp.). Tissue miR-155 expression levels were normalized against the expression of the U6 snRNA 'housekeeping RNA', which was analyzed on the same plates. The TaqMan® primer/probe sets for miR-155 analysis were commercially available from Life Technologies Corp.

5.5.2 RESULTS

[00213] Epididymal white adipose tissue miR-155 expression was significantly elevated in all three analyzed mouse obesity models (hyperphagic db/db and Agouti A^y mutant mice, and high-fat diet-fed $LDLR^{-/-}$ mice) relative to their lean controls (FIG. 13), indicating that there was a strong, model-independent association between increased adiposity and increased adipose tissue miR-155 expression. However, adipose tissue contains multiple cell types. Notably, macrophages, which have previously been shown to express miR-155, have been reported to accumulate in adipose tissue during the progression of obesity. In order to distinguish preadipocyte- and adipocyte-mediated miR-155 expression in adipose tissue, epididymal fat pads were isolated from chow-fed db/db hyperphagic obese and db/m lean mice and chow-fed lean HFD-fed obese $LDLR^{-/-}$ mice, and fractionated to isolate adipocytes, preadipocytes and stromovascular fraction (SVF: a mixture of preadipocytes, endothelial cells, vascular smooth muscle cells, macrophages, *etc.*) cell samples for RT-PCR analyses of miR-155 expression (FIG. 14). In the lean db/m and chow-fed $LDLR^{-/-}$ mice, miR-155 expression was not different among the adipocyte, preadipocyte and SVF samples. However, in obese db/db and HFD-fed $LDLR^{-/-}$ mice, miR-155 expression was markedly increased in the SVF and preadipocyte samples, while there was no change in adipocyte miR-155 expression.

[00214] SVF samples contain preadipocytes and macrophages, both of which are known to express miR-155, as well as other cell types with unknown miR-155 expression potentials. SVF samples were not directly analyzed to determine preadipocyte and macrophage compositions and adipose-resident macrophages were not isolated to quantitate their miR-155

expression; however, RT-PCR analyses for macrophage and endothelial cell markers (CD68 and PECAM-1, respectively) did not detect significant expression differences among SVF fractions isolated from lean and obese mice (FIG. 15), suggesting that SVF miR-155 expression differences were not due to differences in cell composition.

[00215] Similar to SVF samples, no CD68 or PECAM-1 expression differences were detected in preadipocyte samples isolated from lean and obese mice. Moreover, preadipocyte fractions, which were isolated from SVF material, demonstrated <4% of the CD68 and PECAM-1 mRNA expression of their SVF source material, indicating a high degree of macrophage and endothelial cell depletion. Preadipocytes isolated from the adipose tissue of obese mice express 2- to 3-fold more miR-155 than those isolated from lean mice, indicating that there is a marked increase in preadipocyte miR-155 expression in the preadipocytes of obese mice. Based on the observation that increased preadipocyte miR-155 expression attenuates 3T3-L1 and 3T3-F442A adipogenesis *in vitro*, these increases in preadipocyte miR-155 expression are expected to have significant implications for adipogenesis and adipose-related phenotypes during obesity.

[00216] 3T3-L1 preadipocyte miR-155 expression transiently increases 1 to 2 days post-induction, with a maximum 2-fold induction at day 2 (FIG. 8A). Increased adipocyte differentiation is unlikely to explain the miR-155 expression increase found in preadipocyte of obese mice, however, since the 2- to 3-fold increase found in these mice is comparable to that of the maximum induction found in a synchronized population of differentiating adipocytes. Macrophage miR-155 expression is known to be regulated by inflammatory factors. Similarly, adipose resident macrophages, adipose-derived inflammatory factors, and adipose inflammation have been implicated in obesity, suggesting that adipose tissue exposure to inflammatory factors may alter preadipocyte miR-155 expression (see Example 5.6), which may in turn alter preadipocyte function (see Example 5.7)

5.6 EXAMPLE 6 – INFLAMMATORY FACTORS REGULATE MIR-155 EXPRESSION IN 3T3-L1 PREADIPOCYTES AND CORRESPOND WITH MOUSE ADIPOSE MIR-155 LEVELS

[00217] This example describes a study that examined the effect of inflammatory cytokines on 3T3-L1 preadipoctye miR-155 expression, and the correspondence between inflammatory cytokine mRNA and miR-155 expression in isolated mouse and human adipose fractions.

5.6.1 EXPERIMENTAL PROCEDURES

[00218] **Regulation of miR-155 expression in 3T3-L1 adipocytes:** Replica wells of 3T3-L1 preadipocytes grown to confluence in DMEM supplemented with 10% FBS were treated for 24 hours with 0.5, 1, 2, and 5 ng/mL of TNFα or treated with 2 ng/mL TNFα for 2, 4, 10, 24, and 48 hr, and then harvested to isolate total cellular RNA to measure miR-155 expression by RT-PCR. Similarly, replica plates of 3T3-L1 preadipocytes were treated for 24 hr with TNFα (2 ng/mL), LPS (200 ng/mL), IL-1β (5 ng/mL), IFNγ (2 ng/mL), IL-6 (5 ng/mL), or TGFβ (5 ng/mL), or a combination of TNFα (2 ng/mL), IL-1β (5 ng/mL), and IFNγ (2 ng/mL), harvested for total RNA, and analyzed for miR-155 expression by quantitative RT-PCR.

[00219] **Mouse Obesity Models:** $LDLR^{-/-}$, db/m, and db/db mice were obtained from The Jackson Laboratory. Male db/m and db/db male mice were fed standard chow diet (Harlan-Teklad 2920) until sacrificed for tissue collection at 3-months' of-age. Male $LDLR^{-/-}$ mice were fed standard chow diet (Harlan-Teklad 2920) or a high-fat Western diet (RD12079B) for 12 weeks, starting at 10 weeks-of-age. Mice were sacrificed by CO_2 inhalation and immediately dissected to isolate their epididymal fat depots, which flash-frozen in liquid nitrogen or fractionated for SVF isolation essentially as previously described (Halleux *et al.*, 1999). Adipose tissue and isolated tissue fractions were snap-frozen in liquid nitrogen and stored at -80°C until processed for RNA isolation.

[00220] **RT-PCR Analyses:** Total RNA was isolated from 3T3-L1 preadipocytes whole adipose tissue, and adipose tissue adipocyte, stromovascular fractions using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. Tissue miR-155 expression levels were normalized against the expression of the U6 snRNA 'housekeeping RNA', which was analyzed on the same plates. The TaqMan® primer/probe sets for the miR-155 and U6 snRNA RT-PCR analyses are commercially available from Life Technologies Corp.

5.6.2 RESULTS

[00221] Adipose-derived inflammatory factors, and especially TNFα, have been implicated in the development and progression obesity and its associated pathologies. Macrophage expression of miR-155 is regulated by exposure to inflammatory factors, strongly suggesting that adipose tissue exposure to inflammatory factors may similarly induce preadipocyte miR-155 expression and function. In order to test this hypothesis, 3T3-L1

preadipocytes were cultured with 2 ng/mL TNFα for 2-48 hours and analyzed for miR-155 expression. 3T3-L1 preadipocyte miR-155 expression increased 70% after 4 hours exposure to 2 ng/mL TNFα (FIG. 16A) and was induced by as little as 0.5 ng/mL TNFα (FIG. 16B). Similar miR-155 induction results were observed after treatment with lipopolysaccharide, IL-1β, and IFNγ, but not IL-6 or TGF-1β, while a mixture of TNFα, IL-1β, and IFNγ produced a ~4-fold increase (FIG. 17), suggesting that multiple inflammatory pathways act to stimulate miR-155 expression.

[00222] RT-PCR analysis, revealed TNFα, IL-1β, and IFNγ mRNA expression increases in epididymal white adipose tissue and SVF samples of obese db/db *vs.* lean db/m male mice (FIG. 18A) and obese HFD- *vs.* lean chow-fed $LDLR^{-/-}$ male mice (FIG. 18B), consistent with results miR-155 expression in these samples. Based on these results, it appears adipose-derived inflammatory cytokine expression may regulate preadipocyte miR-155 expression to regulate preadipocyte differentiation.

5.7 EXAMPLE 7 – 3T3-L1 PREADIPOCYTE MIR-155 EXPRESSION REGULATES THE RESPONSE TO TNFα EXPOSURE

[00223] This example describes a study to examine the effect of miR-155 knockdown, *via* transfection with a synthetic miR-155 inhibitor oligonucleotide, on TNFα-mediated inhibition of 3T3-L1 preadipocyte differentiation.

5.7.1 EXPERIMENTAL PROCEDURES

[00224] **Cell Culture and Transfection:** Replica wells of 3T3-L1 preadipocytes were transfected for 6 hours with Lipofectamine 2000 (Invitrogen) and 40 pmol of synthetic miR-155 inhibitor or control oligonucleotide (Dharmacon) according to the manufacturer's instructions. Cells were stimulated to differentiate with 3T3-L1 differentiation medium, with or without 2 ng/mL TNFα, and 6 days after induction of differentiation replica plates were stained with Oil Red O to quantitate lipid accumulation or harvested for total RNA isolation to measure expression of the adipocyte marker gene FABP4.

5.7.2 RESULTS

[00225] TNFα has a well-known effect to attenuate 3T3-L1 preadipocyte differentiation. TNFα treatment also induces miR-155 expression, which can also inhibit preadipocyte differentiation. In order to examine the contribution of miR-155 induction to the TNFα-mediated attenuation of adipocyte differentiation, replica wells of 3T3-L1 preadipocytes were

transfected with miR-155 inhibitor or control oligonucleotides then induced to differentiate in the presence or absence of 2 ng/mL TNFα. 3T3-L1 preadipocytes transfected with miR-155 inhibitor revealed increased adipocyte differentiation, as measured by Oil Red O staining of lipid accumulation, either in the presence (FIG. 19A; top *vs.* bottom right) or absence (FIG. 19A; top *vs.* bottom left) of TNFα, and revealed less relative inhibition in response to TNFα treatment than control transfectants. Similar effects were observed upon RT-PCR analysis of FABP4 mRNA expression as a surrogate marker for adipocyte differentiation. FABP4 mRNA expression was ~60% in control *vs.* miR-155 inhibitor transfected 3T3-L1 cells after differentiation in the absence of TNFα (FIG. 19B). TNFα strongly repressed FABP4 mRNA expression (30% control) in 3T3-L1 cells transfected with control oligonucleotide, but this effect was significantly attenuated in treated *vs.* untreated 3T3-L1 cells transfected with miR-155 inhibitor (73% control), resulting in a 4-fold difference in FABP4 mRNA expression between TNFα-treated 3T3-L1 cells transfected with control *vs.* miR-155 inhibitor. These results strongly suggest that miR-155 expression plays a major role in TNFα-mediated inhibition of adipogenesis, which may have major implications for the differentiation and function of preadipocytes, since adipose tissue secretes, and is exposed to, higher concentrations of several inflammatory cytokines that regulate miR-155 expression, including TNFα, during obesity.

5.8 EXAMPLE 8 – 3T3-L1 ADIPOCYTE MIR-155 EXPRESSION REGULATES THE EXPRESSION OF INFLAMMATORY FACTORS

[00226] This example describes a study to examine the effect of miR-155 overexpression, *via* transfection with a synthetic miR-155 mimic oligonucleotide, on pro-inflammatory cytokine expression in mature 3T3-L1 adipocytes.

5.8.1 EXPERIMENTAL PROCEDURES

[00227] **Cell Culture and Transfection:** Replica plates of 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% fetal bovine serum (FBS). Confluent 3T3-L1 cell cultures were then switched to differentiation medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX and 10 μg/mL insulin) to induce adipocyte differentiation. Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were commercially available from Sigma Chemical Co. After 2 days' of culture in differentiation medium, cells were switched to adipocyte culture medium (DMEM supplemented with 10% FBS and 10 μg/mL insulin). Eight days post-induction, replica wells of 3T3-L1 preadipocytes

were transfected for 6 hr with Lipofectamine 2000 (Invitrogen) and 40 pmol of synthetic miR-155 mimic or negative control oligonucleotide (Dharmacon) according to the manufacturer's instructions. After 6 hours, transfected cells were cultured in DMEM supplemented with 10% FBS for an additional 42 hours and then harvested for total RNA isolation.

[00228] **RT-PCR Analyses:** Total RNA was isolated from 3T3-L1 preadipocytes using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies Corp.). RT-PCR expression data for a given mRNA within a sample was normalized against 18S rRNA expression within the same sample, measured on the same reaction plate, as a reference control.

5.8.2 RESULTS

[00229] Adipose tissue is now recognized as an important endocrine organ, and mature adipocytes produce several factors, or adipokines, that are implicated in pathologies associated with obesity. However, while the mechanisms responsible for the regulation of adipocyte expression of these factors are often poorly understood, inflammation is known to regulation the expression of several adipokines. In order to examine the potential role of increased miR-155 expression in mature adipocytes, differentiated 3T3-L1 adipocytes were transfected with miR-155 mimic or control oligonucleotides to determine their effect on the mRNA expression of selected adipokines, lipolytic genes and pro-inflammatory factors. 3T3-L1 preadipocytes transfected with an oligonucleotide mimic of miR-155, demonstrated significantly increased mRNA expression of several genes (FIG. 20), including the pro-inflammatory factors interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS), and the pro-fibrotic factor plasminogen activator inhibitor-1 (PAI-1), the principle inhibitor of tissue plasminogen activator (tPA), and thus a major determinant of the plasma fibrinolytic balance and coagulant potential. No significant changes were detected in any analyzed adipokine or lipid storage genes, indicating that miR-155 expression in mature adipocytes has a predominantly pro-inflammatory phenotype, and may have little or no effect on adipokine production or the expression genes involved in lipid storage or metabolism, at least at the RNA expression level. Endogenous or exogenous miR-155 overexpression may thus result in a proinflammatory adipocyte phenotype, with potential detrimental effects on adipocyte function.

[00230] Conversely, analysis of gene expression in adipose tissue of adult miR-155 KO mice (see Example 5.13), suggested that miR-155 deficiency may increase macrophage

accumulation, but decrease the pro-inflammatory factors such as osteopontin (OPN) and TNFα (FIG. 25A).

5.9 EXAMPLE 9 – HUMAN ADIPOSE TISSUE MIR-155 EXPRESSION INCREASES WITH OBESITY AND CORRELATES WITH PRO-INFLAMMATORY GENE EXPRESSION

[00231] This example describes a study to examine the expression of miR-155 and selected pro-inflammatory factors in paired human subcutaneous and visceral adipose tissue and in preadipocytes, mature adipocytes and SVF samples isolated from these samples, to evaluate potential depot- and cell-type-specific differences in expression and to evaluate potential correlations among miR-155 and pro-inflammatory gene expression.

5.9.1 EXPERIMENTAL PROCEDURES

[00232] **Human Adipose Tissue Isolation and Fractionation:** Visceral and subcutaneous adipose tissue surgical biopsy paired samples were obtained from 14 patients (BMI 23-48 kg/m^2) undergoing abdominal surgery and fractionated essentially as previously described (Halleux *et al.*, 1999).

[00233] **RT-PCR Analyses:** Total RNA was isolated from 3T3-L1 preadipocytes whole adipose tissue, and adipose tissue adipocytes, stromovascular fractions using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. Tissue miR-155 expression levels were normalized against the expression of the U6 snRNA 'housekeeping RNA', which was analyzed on the same plates. The TaqMan® primer/probe sets for the miR-155 and U6 snRNA RT-PCR analyses were commercially available from Life Technologies Corp.

[00234] **Statistical Analyses:** Spearman rank order correlations were used to analyze correlation among RT-PCR and biometric data. Mann Whitney rank sum nonparametric tests were used to analyzed RT-PCR data for significant differences in miR-155 or gene expression in SC and V adipose tissue, or adipocytes, preadipocytes or SVF samples derived from SC or V adipose tissue.

5.9.2 RESULTS

[00235] Comparison of subcutaneous (SC) and visceral (V) preadipocyte miR-155 expression with mRNA expression and other phenotype data using Spearman's rank order correlation analyses revealed that miR-155 expression in SC and V preadipocytes similarly

correlated with BMI (V: r = 0.66, SC: r = 0.64), corresponding to results from obese mouse models where increased preadipocyte miR-155 expression was detected in obese mice (Example 5.2). Moreover, miR-155 expression was significantly correlated among SC and V adipose tissue depots (r = 0.69) and preadipocytes (r = 0.78), suggesting that similar systemic or regional processes regulate miR-155 expression at different anatomical sites. Preadipocytes isolated from V *vs.* SC adipose tissue demonstrated similar miR-155 expression. However, further analyses revealed fat-depot-specific correlation differences with inflammatory cytokine expression, where preadipocyte miR-155 expression more closely correlated with IL-1 and TNFα mRNA expression in V *vs.* SC adipocytes (IL-1, V: r = 0.62 *vs.* SC: r = 0.40; TNFα, V: r = 0.52 *vs.* SC: r = 0.40), and significantly correlated only with monocyte chemotactic protein-1 (MCP-1) expression of visceral adipocytes (r = 0.48). Expression of CD68, a macrophage marker, and expression of IL-1β, TNFα and MCP-1 did not significantly differ among SC and V preadipocyte samples, suggesting that miR-155:cytokine correlation differences among these samples were not due to differences in preadipocyte purity or exposure to preadipocyte-derived pro-inflammatory factors.

[00236] SVF cells also express several pro-inflammatory factors that induce miR-155 expression in cultured 3T2-L1 preadipocytes, including IL-1β, TNFα and MCP-1. SC and V preadipocytes may therefore either respond differently to similar stimuli and/or differences in gene expression in other cell types may account for the V and SC preadipocyte correlation differences. SC and V adipocytes revealed similar MCP-1, TNFα and IL-1β mRNA expression levels, but SVF cells isolated from V adipose tissue expressed significantly more TNFα (3.3-fold) and IL-1β (1.8-fold) than those isolated from SC adipose, which could partially explain correlation differences among SC and V preadipocyte miR155 and pro-inflammatory gene expression. Indeed SC preadipocyte miR-155 expression more closely correlated with SVF vs. preadipocyte IL-1β expression (r = 0.56 *vs.* 0.40) and TNFα expression (r = 0.51 *vs.* 0.40).

[00237] Similar to results from mouse studies (Example 5.2) and cell culture studies (Example 5.7), it thus appears that adipose, and specifically preadipocyte, miR-155 expression increases with increased adiposity, and that preadipocyte miR-155 expression correlates with adipose-associated proinflammatory gene expression. Moreover, body mass index, a surrogate measure of adiposity, significantly correlated with proinflammatory cytokine expression in all SC and V adipocyte and SVF fractions (IL-1: r = 0.58-0.82; TNFα: r = 0.54-0.82; MCP-

1: r = 0.42-0.60), suggesting that increased adipose-derived cytokine expression is responsible for the correlation between miR-155 and BMI.

[00238] In summary, this example indicates that miR-155 expression is similar, and appears to experience similar regulation, in anatomically distinct adipose tissue depots, and that adipocyte- and adipose-derived proinflammatory factors, including IL-1β, TNFα and MCP-1, may play a significant role in the regulation of human miR-155 expression.

5.10 EXAMPLE 10 – C/EBPβ EXPRESSION REGULATES MIR-155 EXPRESSION IN 3T3-L1 PREADIPOCYTES

[00239] This example describes a study to examine the effect of C/EBPβ overexpression on miR-155 expression in 3T3-L1 preadipocytes.

5.10.1 EXPERIMENTAL PROCEDURES

[00240] **Silencer RNA (siRNA) Transfection:** Replica 12-well plates of 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% fetal bovine serum (FBS) and then transfected with 20 pmol of a control siRNA, or siRNA specific for C/EBPα, C/EBPβ, or KLF5 (Qiagen) for 6 hours with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 24-hours' transfection, cells were switched to 3T3-L1 adipocyte differentiation medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX and 10 μg/mL insulin) and after 24-hours' culture in differentiation medium, cells were harvested for RNA isolation.

[00241] **Recombinant C/EBPβ Expression:** Retrovirus sources, construction and amplification procedures for pBABE-puro, pBABE-puro-miR155, pBABE-hygro and pBABE-hygro-LAP2 are as described above. 3T3-L1 preadipocytes were co-infected with puromycin-selectable control (pBABE-puro) or miR-155-overexpressing (pBABE-puro-miR155) retrovirus and hygromycin-selectable control (pBABE-hygro) or C/EBPβ-overexpressing (pBABE-hygro-LAP2) retroviruses for 14 days in DMEM supplemented with 10% FBS, 2 μg/mL puromycin (InvivoGen) and 400 μg/mL hygromycin (InvivoGen), and then harvested to isolate total cellular RNA for RT-PCR analysis of miR-155 expression.

5.10.2 RESULTS

[00242] 3T3-L1 preadipocyte C/EBPβ mRNA expression is markedly increased as early as 2 hours after induction of adipocyte differentiation (FIG. 10), well before significant increases in miR-155 expression observed at 1 to 2 days post-induction (FIG. 6). Similarly,

decreases in miR-155 expression lags behind decreases in C/EBPβ protein expression. C/EBPβ is one of several regulatory transcription factors (including *e.g.*, C/EBPα and KLF5) that plays an important role in adipogenesis. Since direct and indirect feedback regulation mechanisms are not uncommon in a number of biological processes, and since little is known about the regulation of miR-155 expression, the ability of C/EBPα, C/EBPβ and/or KLF5 to regulate miR-155 expression was examined by determining the effect of siRNA-mediated C/EBPα, C/EBPβ and/or KLF5 repression to alter miR-155 expression in 3T3-L1 preadipocytes. As shown in FIG. 21A, only C/EBPβ siRNA treatment significantly repressed miR-155 expression at 1 day post-induction, suggesting that increased C/EBPβ expression during early adipogenesis may either directly or indirectly induce miR-155 expression. Conversely, retroviral-mediated C/EBPβ overexpression strongly induced miR-155 expression (FIG. 21B), further suggesting a functional association between C/EBPβ and miR-155 expression. Equal or greater miR-155 induction was observed in 3T3-L1 preadipocytes infected with retrovirus expressing miR-155, indicating that these cells have not overloaded their capacity to express this miRNA.

[00243] C/EBPβ-mediated induction of miR-155 expression suggests an alternate explanation for the failure of C/EBPβ overexpression to completely rescue miR-155 inhibition (see Example 5.3), since, based on these results, C/EBPβ overexpression would be expected to further increase miR-155 expression and thus further inhibit endogenous C/EBPβ expression or the expression of an as-yet-unidentified second miR-155 gene target.

5.11 EXAMPLE 11 – MIR-155 DEFICIENCY INCREASES ADIPOGENESIS IN CULTURED MOUSE EMBRYONIC FIBROBLASTS OF MIR-155 KNOCKOUT *VS.* WILD-TYPE MICE

[00244] This example describes a study to examine the effect of miR-155 deficiency on the adipogenic differentiation potential of mouse embryonic fibroblasts during primary culture.

5.11.1 EXPERIMENTAL PROCEDURES

[00245] **Mice:** C57B/6J wild-type mice were purchased from The Jackson Laboratory; breeding pairs of miR-155 KO mice on a C57B/6J genetic background were obtained from Dr. Antony Rodriguez (Baylor College of Medicine, Houston, TX, USA), and heterozygous miR-155 KO mice (miR-155$^{+/-}$) were generated by crossing C57B/6J mice with homozygous miR-155 KO mice.

[00246] **Mouse Embryonic Fibroblast (MEF) Generation:** Heterozygous miR-155 KO mice were crossed to generate wild-type, miR-155$^{+/-}$ and miR-155$^{-/-}$ embryos. MEFs were isolated from E12.5 littermate embryos and cultured at 37°C in DMEM supplemented with 10% FBS (Invitrogen/Life Technologies Corp.). After three passages, cells were plated into 6-well culture dishes and propagated to confluence. Two days after reaching confluence, cell cultures were induced to differentiate into adipocytes by culture in DMEM supplemented with 0.5 mM IBMX, 1 μM dexamethasone, 5 μg/mL insulin, 10% FBS, and 0.5 μM rosiglitazone (Cayman Chemical Company). After 48 hours induction, the culture medium was replaced with DMEM containing 5 μg/mL insulin and 0.5 μM rosiglitazone, which was replaced every other day. Replica MEF cultures were harvested at day 10 post-induction and stained with Oil Red O to detect cytoplasmic lipid accumulation, as a marker of adipocyte differentiation, or to isolate total cellular RNA for RT-PCR analysis.

[00247] **RT-PCR Analyses:** Total RNA was isolated from MEF cultures using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Invitrogen/Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. Tissue miR-155 expression levels were normalized against the expression of the U6 snRNA 'housekeeping RNA', which was analyzed on the same plates. The TaqMan® primer/probe sets for miR-155 analysis were also purchased from Invitrogen/Life Technologies Corp.

5.11.2 RESULTS

[00248] Mouse embryonic fibroblasts (MEFs) isolated from miR-155 deficient *vs.* wild-type littermate mice demonstrated a modest increase in Oil Red O staining of lipid accumulation after 10 days of *in vitro* culture (FIG. 22A). RT-PCR analysis of these differentiated MEF cultures also revealed a ~1.5-fold increase in the expression of adipogenic marker genes in mir-155 deficient *vs.* wild-type cells (FIG. 22B). Taken together, these results suggest that miR-155 deficiency slightly enhanced the adipogenic potential of primary MEFs. Similar results were obtained with differentiating 3T3-L1 preadipocytes made miR-155 deficient by transfection with a synthetic miR-155 inhibitor (see Example 5.2), suggesting that 3T3-L1 preadipocytes miR-155 effects are also operative in during the differentiation process of primary mouse adipocyte cells.

5.12 EXAMPLE 12 – NEWBORN MIR-155 KO HAVE MORE BODY FAT, LARGER ADIPOCYTES AND INCREASED ADIPOSE EXPRESSION OF SEVERAL ADIPOCYTE-RELATED GENES

[00249] This example describes a study to examine the effect of whole-body miR-155 deficiency on body composition, adipocyte size, and adipose tissue gene expression.

5.12.1 EXPERIMENTAL PROCEDURES

[00250] **Body composition and Tissue collection:** Littermate mice from heterozygous miR-155 mouse parental crosses were collected at 3 days after birth, weighed, and then analyzed for body composition by NMR using an EchoMRI 3-in-1 quantitative MRI system (Echo Medical Systems, Houston, TX, USA). After these measurements, mice were anesthetized by isoflurane inhalation, and sacrificed by decapitation just below the skull. Immediately after sacrifice, mice were placed in ice-cold PBS and dissected to isolate anatomical regions and tissue samples. Mice were first bisected at the level of the lower rib cage, and the neck and chest region was placed into formalin until processed for histological analysis, then the abdominal cavity was opened and dissected to remove the epididymal fat pad, which was flash-frozen in liquid nitrogen and stored at -80°C until processed for RNA isolation. Finally, tail samples for genotyping were collected from each mouse and frozen at -80°C until processed for DNA isolation.

[00251] **Mouse genotyping:** Genomic DNA was isolated from tail samples using a DNeasy Blood & Tissue Kit (Qiagen), and the miR-155 genomic DNA loci in each Genomic DNA sample were PCR amplified and size-fractionated by agarose gel electrophoresis to distinguish homozygous (miR-155$^{-/-}$), heterozygous (miR-155$^{+/-}$), and wild-type littermate mice. PCR reactions were performed with *Thermus aquaticus* (*Taq*) polymerase (Qiagen), a sense-strand miR-155 genomic DNA locus specific primer (5′-GCCCTGGCTGTACCCCCTATCTTG-3′) (SEQ ID NO:42) and two antisense-strand DNA primers (5′-GAAATGCGTAGGAAACGTGGGTCTC-3′) (SEQ ID NO:43) and (5′-CATACAGCCTTCAGCAAGCCTCCA-3′) (SEQ ID NO:44), in which the wild-type miR-155 allele produced a 564-bp fragment and the KO miR-155 allele yielded a 450-bp fragment.

[00252] **Histology:** Epididymal white adipose tissue samples were fixed overnight in 10% formalin at 4°C, then ethanol dehydrated, embedded in paraffin, sectioned in 6-μm slices, and then hematoxylin and eosin stained to resolve cellular structures. Mature adipocytes in these sections were identified by their characteristic appearance, and maximum adipocyte diameters were measured using NIS-Elements AR 3.0 Software (Nikon Instruments, Inc.,

Melville, NY, USA) using a procedure in which the same operator manually traced the maximum diameter of ≥150 adipocytes per sample.

[00253] **RT-PCR Analyses:** Total RNA was isolated from epididymal fat tissue samples of 3-day-old miR-155 and wild-type mice using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies Corp.), and mRNA expression in each sample was normalized against 18S rRNA in the same sample. RT-PCR mRNA and 18S rRNA samples were analyzed on the same plates. The TaqMan® primer/probe sets for all mRNAs and 18S rRNA were purchased from Life Technologies Corp.

5.12.2 RESULTS

[00254] Characteristic adipocyte morphology develops during the first 24 hr after birth, with little or no adipocyte accumulation at birth (Ailhaud *et al.,* 1992). After this rapid development, however, differentiation of adipocytes from preadipocytes is believed to continue throughout the lifetime of an animal (Rosen, 2002). NMR analysis of body composition revealed that 3-day-old mice miR-155 KO mice had significantly more percent body fat than their wild-type littermate controls (FIG. 23A: 6.3% *vs.* 5.1%, respectively), despite there being no difference in body mass between these groups, suggesting that newborn miR-155 KO mice may preferentially gain more fat mass. Histological analysis also detected a significant increase in subcutaneous adipocyte diameter in miR-155 KO *vs.* wild-type mice (FIG. 23B: 28.9 μm *vs.* 16.9 μm, respectively), suggesting that adipocytes of miR-155 KO and wild-type littermate mice display function differences in energy storage. RT-PCR analysis of gene expression in the epididymal white adipose tissue of these mice (FIG. 23C) also revealed significant, or near-significant, increases in several adipogenesis-associated genes (C/EBPα, C/EBPβ, and PPARγ) or lipid metabolism related genes (HSL and FABP4) in miR-155 KO *vs.* wild-type mouse adipose. Taken together, these results suggest that miR-155 KO mice develop larger and more metabolically active adipose tissue depots, during a well-defined period of post-natal adipose differentiation, and that increased adipose mass and metabolic activity result from effects of miR-155 on adipocyte differentiation and function.

5.13 EXAMPLE 13 – ADULT MIR-155 KO ADIPOSE TISSUE REVEALS ALTERED EXPRESSION OF ADIPOGENIC, LIPOLYTIC, INFLAMMATORY AND METABOLIC GENES

5.13.1 EXPERIMENTAL PROCEDURES

[00255] **Mice:** C57B/6J wild-type mice were purchased from The Jackson Laboratory, breeding pairs of miR-155 KO mice on a C57B/6J genetic background were obtained from Dr. Antony Rodriguez (Baylor College of Medicine), and heterozygous miR-155 KO mice were generated by crossing C57B/6J mice with homozygous miR-155 KO mice. Littermate miR-155 KO and wild-type male mice were obtained from heterozygous miR-155 mouse parental crosses and fed standard chow diet (Harlan-Teklad 2920) until sacrificed for tissue collection at 10 weeks-of-age. Mice were genotyped, as described below, from a tail snip biopsy obtained at weaning, and littermate wild-type and miR-155 KO male mice were group-housed until sacrifice. Mice were sacrificed by CO_2 inhalation and, immediately after sacrifice, their epididymal fat depots were isolated, snap-frozen in liquid nitrogen and stored at -80°C until processed for whole-tissue RNA isolation.

[00256] **Mouse genotyping:** Genomic DNA was isolated from tail samples using DNeasy Blood & Tissue Kits (Qiagen), and the miR-155 genomic DNA loci in each genomic DNA sample were PCR amplified and size-fractionated by agarose gel electrophoresis to distinguish homozygous (miR-155$^{-/-}$), heterozygous (miR-155$^{+/-}$), and wild-type littermate mice. PCR reactions were performed with *Taq* polymerase (Qiagen), a sense-strand miR-155 genomic DNA locus specific primer (5′-GCCCTGGCTGTACCCCCTATCTTG-3′) (SEQ ID NO:45) and two antisense-strand DNA primers (5′-GAAATGCGTAGGAAACGTGGGTCTC-3′) (SEQ ID NO:46) and (5′-CATACAGCCTTCAGCAAGCCTCCA-3′) (SEQ ID NO:47), in which the wild-type miR-155 allele produced a 564-bp fragment and the KO miR-155 allele yielded a 450-bp fragment.

[00257] **RT-PCR Analyses:** Total RNA was isolated from whole adipose tissue, and adipose tissue adipocyte, preadipocyte and stromovascular fractions using TRIzol Reagent (Invitrogen), and reverse-transcribed to cDNA using a TaqMan® miRNA Reverse Transcription Kit (Life Technologies Corp.). Quantitative RT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. Tissue mRNA expression levels were normalized against the expression of 18S rRNA, a 'housekeeping RNA', which was analyzed on the same plates. TaqMan® primer/probe sets for all mRNA analyses were purchased from Life Technologies Corp.

5.13.2 RESULTS

[00258] RT-PCR analysis of adipose tissue isolated from littermate miR-155 KO and wild-type male mice detected several significant gene expression differences. Several adipogenesis-related genes were more highly expressed in miR-155 KO adipose tissue, including PPARγ (1.2-fold), C/EBPα (2-fold) and C/EBPβ (1.7-fold), although not all these differences reached statistical significance (FIG. 24A). Expression of FABP4, a lipid transport protein, and fatty acid synthase were not significantly different between the two groups, suggesting that there were no differences in fatty acid uptake or *de novo* synthesis. However, miR-155 KO adipose demonstrated increased expression of two genes that regulate cellular triacylglyerol levels and thus regulate cellular lipid accumulation, glycerol-3-phosphate acyltransferase (GPAT) and diglyceride O-acyltransferase (DGAT). As shown in FIG. 24B, GPAT, which catalyzes the initial commitment step in glycerolipid biosynthesis, was increased 2.4-fold, while DGAT, which catalyzes the final commitment step in triacylglyceride synthesis, was increased 1.5-fold, although this difference did not reach statistical significant ($p = 0.07$). GPAT and DGAT activity increases would tend to promote increased lipid accumulation. However, miR-155 adipose also demonstrated increased expression of the lipolytic enzymes adipose triglyceride lipase (ATGL; 1.8-fold) and hormone sensitive lipase (HSL; 1.3-fold), which catalyze the first and second steps in triacylglyceride lipolysis. Moreover, miR-155 KO adipose also revealed markedly increased expression of two fatty acid β-oxidation genes (FIG. 24C), hydroxyacyl-Coenzyme A dehydrogenase α (HADHA; 1.8-fold) and acyl-Coenzyme A dehydrogenase medium chain (ACADM; 1.7-fold), suggesting that miR-155 KO adipose has increased β-oxidation capacity. Carnitine palmityol transferase 1 (CPT1), which regulates fatty acid transport into mitochondria and thus directly regulates the rate of fatty acid β-oxidation, has three known isoforms, each of which displays preferential tissue expression patterns. CPT1b is the major CPT1 isoform expressed in adipose tissue. As shown in FIG. 24C, adipose CPT1b expression was not different between miR-155 KO and wild-type mice, while miR-155 KO mice revealed a slight decrease in adipose tissue CPT1a expression (0.7-fold). CPT1 activity is regulated by acyl-CoA carboxylase 1 and 2 (ACC1 and ACC2) activity, but neither ACC1 nor ACC2 expression differed between these groups, suggesting that miR-155 mouse adipose might have a reduced capacity to β-oxidize fatty acids due to an unopposed decrease in CPT1a expression. Taken together this data suggested that miR-155 KO adipose synthesized more triacylglyceride from dietary free fatty acid, while at the same time hydrolyzing more triacylglyceride to fatty acid. It is unclear, however, whether decreased

CPT1a mRNA expression significantly decreases fatty acid transport to limit β-oxidation in the face of increased HADHA and ACADM expression.

[00259] Both inflammation and miR-155 expression can regulate adipose function, and several inflammation-related gene expression differences were detected among miR-155 KO and wild-type adipose tissue (FIG. 25A). CD68, a marker of macrophage accumulation, was 1.4-fold increased in miR-155 adipose. However, despite this increase, miR-155 adipose demonstrated no significant increases in IL-1β, MCP-1, PAI-1, IL-6, or iNOS expression, and instead demonstrated marked reductions in osteopontin (OPN; 0.12-fold) and TNFα (0.7-fold) expression, signifying a potential decrease in the inflammatory state of miR-155 adipose tissue.

[00260] Adipose tissue is an endocrine organ, secreting factors several factors that have important metabolic and physiologic functions. Adult miR-155 KO mouse adipose expressed significantly more resistin and angiotensinogen than that of wild-type mice and a tendency to express more adiponectin. In rodents, resistin is expressed primarily from adipocytes and is upregulated in response to insulin and glucose; it also suppresses insulin signaling. However, in humans resistin is primarily expressed by macrophages, where it seems play role in immune cell recruitment and pro-inflammatory cytokine secretion. Rodent resistin data is thus unlikely to translate into human studies. Similarly, while angiotensinogen is required for adipose tissue development and plays an important role in blood pressure regulation, but angiotensinogen overexpression has not been shown to alter either process, suggesting that differential expression in wild-type and miR-155 KO adipose tissue is unlikely to have a systemic effect. Adiponectin demonstrates adipocyte-specific expression, is the most highly expressed mRNA in mature adipocytes, and has well-documented systemic effects to reduce inflammation, including TNFα expression, and increase insulin sensitivity. However, adiponectin mRNA expression increases in miR-155 KO vs. wild-type adipose did not reach was not significance, and may not be reflected at the protein level.

5.14 EXAMPLE 14 – MIR-155 KO MICE EXHIBIT CHANGES IN INNATE AND DIET-INDUCED ADIPOGENESIS *VS.* WILD-TYPE MICE

[00261] In order to examine the role of miR-155 expression on *in vivo* adipogenesis nuclear magnetic resonance (NMR) was used to examine the body fat composition of littermate wildtype and miR-155-deficient mice. Since mice develop characteristic adipocyte morphology during the first 24 hours after birth, body fat 3 days after birth was examined to determine the effect of miR-155 KO during this period of near maximal adipocyte differentiation.

5.14.1 Experimental Procedures

[00262] **Mice:** C57B/6J wild-type mice were obtained from The Jackson Laboratory). Breeding pairs of miR-155 KO mice on a C57B/6J genetic background (Dr. Antony Rodriguez, Baylor College of Medicine, Houston, TX, USA), and heterozygous miR-155 KO mice were generated by crossing C57B/6J mice with homozygous miR-155 KO mice. Wild-type and miR-155 KO male mice were fed standard low-fat chow diet (Harlan-Teklad 2920) from weaning until 10 weeks of age at which time each genotype as assorted into two groups one of which was switched to a high-fat, high-sugar "Western" diet (RD 12079B) while the other was maintained of standard low-fat chow diet (Harlan-Teklad 2920) for the duration of the study. One week prior to starting mice on diet, mice were weighed and NMR-analyzed for body composition using a EchoMRI 3-in-1 quantitative MRI system (Echo Medical Systems), and then reweighed and reanalyzed by NMR after 1, 2, 3, 4, 5, 6, 7, and 8 weeks on diet. Mice were analyzed for insulin sensitivity by intraperitoneal tolerance test after 10 weeks on diet. Mice were then allowed to recover until 12 weeks on diet, at which time they were reweighed and reanalyzed by NMR, and sacrificed for tissue collection. Fasting plasma and insulin samples were used to calculate HOMA-IR values of insulin sensitivity.

[00263] **ITT Assay:** After 10 weeks on diet, mice were fasted for 6 hours and baseline glucose samples were obtained by nicking their tail tips. Mice were then injected intraperitoneally with 0.5 IU/kg insulin and blood samples were collected at 15, 30, 60 and 90 minutes' post-injection. HOMA-IR values were computed using fasting glucose and insulin measurements obtained after 12 weeks on the diet.

5.14.2 Results

[00264] In order to test the effect of miR-155 deficiency upon adipose tissue development, miR-155 KO and wild-type control mice were fed HFD to induce fat deposition. Body weights of miR-155 KO and wild-type mice were not different at baseline (FIG. 27A), but miR-155 KO gained significantly more weight than wild-type mice when fed HFD (13%, 37.2g *vs.* 32.9g), despite similar food intake. NMR measurement of body composition revealed that miR-155 mice had modestly more body fat than wild-type mice at baseline (FIG. 27C, 1.8g *vs.* 1.2g), but gained markedly more fat after 8 weeks of HFD (75%, 9.3g *vs.* 5.3g), suggesting that miR-155 deficiency enhanced adipogenesis in the miR-155 KO mice. Analysis of longitudinal weight and body composition changes in individual mice revealed a similar pattern, with significant differences in body and fat mass detectable after 3 weeks of HFD (FIG. 27B and FIG. 27D). NMR-determined lean body mass was not significantly different in

miR-155 KO *vs.* wild-type mice at baseline or after 8 weeks of HFD, but miR-155 KO demonstrated significant or borderline significant differences in lean body mass vs. wild-type mice for the first four weeks after HFD (FIG. 27E), which corresponded to modest decreases in lean body mass detected in the first 2 weeks of HFD (FIG. 27F). Body and mass differences between miR-155 KO and wild-type mice were readily detectable by eye (FIG. 28).

[00265] Similar studies were conducted with miR-155 KO and wild-type control mice fed standard chow diet to assess the effect of miR-155 KO on adipogenesis in mice fed a healthy, low-fat diet. Body weights of miR-155 KO and wild-type mice were not different at baseline or after 8 weeks of chow diet (FIG. 29A and FIG. 29B). However, despite similar initial body compositions, wild-type mice gained significantly more fat mass than miR-155 KO mice (FIG. 29C and FIG. 29D), since miR-155 KO did not gain significant fat mass over this time course, suggesting that miR-155 deficiency attenuates age-associated increases in fat mass in mice fed a standard chow diet. Moreover, miR-155 deficient mice also demonstrated a modest but non-statistically-significant trend towards increased lean mass vs. wild-type mice. Differences in lean and fat mass in miR-155 KO *vs.* wild-type mice are similar and may account for the similar body weights and weight gains of miR-155 KO and wild-type mice. For example, after 8 weeks of chow diet, miR-155 KO vs. wild-type mouse fat mass differences (-1.30 g: 1.66g *vs.* 2.96 g) very closely parallel lean mass differences (1.26 g: 26.96 g *vs.* 25.70 g), suggesting that miR-155 KO mice may accumulate lean body mass in place an age-associate fat gain observed in wild-type mice. In order to evaluate this theory, miR-155 KO and wild-type mice were dissected to isolate an easily isolated skeletal muscle group, the gastrocnemius, to analyze a potential genotype-specific effect on lean muscle mass. Similar to differences in NMR-determined lean body mass measurements, miR-155 KO *vs.* wild-type mice also demonstrated a trend towards increased gastrocnemius mass per body weight (0.338% *vs.* 0.320%, Student's *t*-test $p = 0.12$, $n = 6$/group; 5.6% increase) that closely paralleled the previously observed difference in lean mass per body weight (90.2% *vs.* 86.1%, Mann Whitney $p = 0.09$; 4.4% increase).

[00266] Insulin tolerance tests (ITTs) were performed to address the effect of genotype and diet on insulin sensitivity. Chow-fed miR-155 KO demonstrated similar insulin responses (FIG. 29A), while HFD-fed miR-155 KO mice demonstrated modestly increased insulin resistance *vs.* their diet- and age-matched wild-type controls. HOMA-IR measurements of insulin resistance, however, detected a significant difference among all groups. Similar to ITT data, HFD-fed miR-155 KO mice were found to be more insulin resistant than their wild-type

controls. HFD-fed miR-155 KO, but not wildtype, mice were also more insulin-resistant than their chow-fed controls. ITT and HOMA-IR difference between HFD-fed miR-155 KO and wild-type mice may be explained by the increased adiposity of the miR-155 KO mice, since adiposity is closely linked to insulin resistance. Surprisingly, however, chow-fed miR-155 KO mice were significantly more insulin-sensitive than their wild-type controls despite having similar body weights, and only modest differences in fat and lean mass. Body composition may contribute to this difference, since wild-type mice have more fat mass while miR-155 KO mice tend to have more lean mass, and fat and muscle have opposite effects on systemic insulin sensitivity. However, additional mechanisms may be responsible for this observation.

5.15 EXAMPLE 15 – METHODS FOR LOCALIZING MIR-155 COMPOSITIONS TO SELECTED SITE(S) WITHIN THE BODY OF AN ANIMAL

[00267] The present example describes an exemplary animal model protocol for assaying the effectiveness of miR-155 compositions in regulating the development or reduction of fat cells in one or more selected tissue sites within the body of a mammal. In this study, microspheres may utilized to selectively deliver the miR-155 compositions to localizations of fatty tissue through the use of one or more targeting modalities, including, without limitation, high frequency ultrasound (HIFU)-mediated localization. Such methods represent experimental evidence of the success when using miR-155-specific compositions to decrease adiposity within the animal.

5.15.1 EXPERIMENTAL PROCEDURES

[00268] In exemplary studies, mice are induced using 3-4% isoflurane in the designated induction chamber, prior to being set up for the procedure. Each animal is then taped plastic platform and partially submerged in a shallow pan of warm (*e.g.,* 35°C) degassed water for coupling to a therapeutic transducer (Philips TIPS system). Ultrasound B-mode imaging is used to provide anatomical targeting. A warming pad under the bath assists in maintaining the temperature during the procedure. Ultrasound may be used in either of two ways, first as an alternative means of raising the local temperature (*e.g.,* acoustic power levels up to 10 W at up to 80 +ACU- duty cycle for 10 min), or as a mechanical stimulation to increase local tissue permeability (power up to 40 W at 5+ACU- duty cycle and 1 Hz repetition rate for 100 pulses per sonication zone). Following treatment and recovery, the mouse is injected *via* tail vein with non-toxic carboxylated fluorescent microspheres of various sizes from 20 to 200 nm diameter FluoSpheres® (Invitrogen/Life Technologies Corp.), for optical imaging of delivery to the

adipose tissue. Previous studies have shown no morbidity in mice at up to 200 microliters of stock solution (Richer *et al.,* 2000). The example described here is useful in determining if microspheres can be targeted to one or more regions of fat accumulation in the animal's body using high frequency ultrasound (HIFU) as a method for delivering selected miR-155 compositions to alter, and preferably decrease adiposity in the one or more regions. The mice are dried off with a towel and kept on a water circulating heating pad until they are completely dry. They are monitored at regular intervals during and after recovery until they are completely dry.

[00269] Following injection, the animals may be imaged under general anesthesia in the Xenogen system at regular intervals of greater than 4 hours. Animals are then euthanized within 48 hours of treatment, and the treated region resected for histology. During the interim period, the mice are visually checked twice daily for signs of pain or infection, such as decreased use of limb, visible lesions, gnawing at the treated area, guarding of the area, decreased mobility, or decreased appetite. If pain is suspected using the criteria above, the animal may be treated with buprenorphine, 0.05 mg/kg given subcutaneously, or humanely euthanized.

5.16 EXAMPLE 16 – DELIVERY METHODS

[00270] Systemic miR-155 KO has significant *in vivo* effects on adipose tissue development and function. Various delivery methods are useful for modulating adipose miR-155 activity through direct modulation of miR-155 activity or miR-155 target activity (*i.e.,* C\EBPβ). In certain embodiments, optimized miR-155 derivative sequences may be utilized to exert greater, and/or more selective effects on adipocyte differentiation, including, for example, RNA phosphodiester backbone modifications to improve the stability of these agents. Systemic injections may be performed with naked or encapsulated miR-155 derivatives or scrambled controls to determine the relative toxicity of the specific molecules and the delivery systems (*e.g.,* liposomes, dendrimers, *etc.*) and the effective dose required to significantly alter miR-155 activity or miR-155 target activity in adipose tissue, adipose cell fractions, and non-adipose tissues. Similar studies utilizing vector constructs that express miR-155-derived sequences (*e.g.,* plasmids, transposons, *etc.*) may facilitate improved or enhanced biological effects of the miR-155 compositions disclosed herein as compared to that observed using only systemic small RNA molecule delivery approaches. Site-specific delivery using direct injection in subcutaneous adipose tissue specific and selected visceral adipose tissue depots represents one method in which the dose required to significantly alter miR-155 activity or miR-155 target

activity for a particular regimen may be determined. Similarly, the use of tissue- or cell-targeted delivery methods (including, for example HIFU-mediated nanoparticle delivery and such like) is also contemplated for improving and/or optimizing particular dosing regimens and/or treatment modalities.

5.17 EXAMPLE 17 – EXEMPLARY MIR-155 COMPOSITIONS

[00271] In the practice of the invention, a variety of miR-155 compositions, as well as analogs, antagonists, or agonists thereof, may be useful in one or more of the therapeutic regimens described herein.

[00272] Examples of such sequence include, without limitation, the following oligonucleotide compositions depicted in FIG. 32A:

[00273] *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Pongo pygmaeus* (Bornean orangutan), *Macaca mulatta* (Rhesus monkey), *Canis familiaris* (dog), *Bos taurus* (cow), *Equus caballus* (horse) and *Ornithorhynchus anatinus* (platypus) miR-155 mature sequences are all identical:

UUAAUGCUAAUCGUGAUAGGGGU (SEQ ID NO:1);

[00274] The *Mus musculus* (mouse) miR-155 mature sequence differs from these by a single C to U change at position 12:

UUAAUGCUAAUUGUGAUAGGGGU (SEQ ID NO:2);

[00275] The *Gallus gallus* (chicken), *Danio rerio* (zebrafish), *Taeniopygia guttata* (Zebra finch), and *Xenopus tropicalis* (Western clawed frog), miR-155 sequences are all identical:

UUAAUGCUAAUCGUGAUAGGGG (SEQ ID NO:3),

and are highly conserved with SEQ ID NO:1 and SEQ ID NO:2.

[00276] Other non-mammalian species also have conserved miR-155 sequences as well:

Ciona intestinalis (vase tunicate):

UUAAUGCUAAUAGUGAUAGGG (SEQ ID NO:4); and

Ciona savignyi (Pacific sea squirt) miR-155 mature sequence:

UUAAUGCUAAUAAGUGAUUUAUG (SEQ ID NO:5).

[00277] The comparatively high homology of these sequences is shown graphically in FIG. 32B.

5.18 EXAMPLE 18 – SELECTION OF MIRNA-155 AGONISTS AND ANTAGONISTS

[00278] Mature miR-155 sequence demonstrates strong evolutionary conservation. As shown above, the human miR-155 sequence (5′-UUAAUGCUAAUCGUGAUAGGGGU-3′) (SEQ ID NO:1) is perfectly conserved in the mature miR-155 transcripts of chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), platypus (*Ornithorhynchus anatinus*) and domestic dog (*Canis familiaris*), horse (*Equus caballus*) and cow (*Bos taurus*), while the mouse (*Mus musculus*) mature miR-155 transcript (5′-UUAAUGCUAAUUGUGAUAGGGGU-3′) (SEQ ID NO:2) differs from the human miR-155 sequence by a single nucleotide, due to a cytosine to uracil nucleotide substitution at the 12th nucleotide position of the mature mouse sequence. Thus, miR-155 is highly conserved in all mammals for which miR-155 sequence is available.

[00279] Non-mammalian chordates also display strong miR-155 sequence conservation, since chicken (*Gallus gallus*), zebrafish (*Danio rerio*) and pipid frog (*Xenopus tropilcalis*) mature miR-155 transcripts, are identical (5′-UUAAUGCUAAUCGUGAUAGGGG-3′) (SEQ ID NO:3) and differ from the mature human miR-155 sequence only due to the loss of the 3' terminal uracil at the 23rd nucleotide position of the mature human miR-155 sequence. Non-chordates also display some degree of miR-155 sequence conservation with mature human miR-155 sequence, however to a variable degree. The mature miR-155 sequence of the sea squirt *Ciona intestinalis* (5′-UUAAUGCUAAUAGUGAUAGGG-3′) (SEQ ID NO:4) differs from mature human miR-155 sequence only due a cytosine to adenine substitution at the 12th nucleotide of the mature miR-155 sequence and the loss of two 3′ terminal nucleotides. However, the mature miR-155 sequence of the sea squirt *Ciona savignyi* (5′-UUAAUGCUAAUAAGUGAUUUAUG-3′) (SEQ ID NO:5) displays much less sequence conservation, sharing only the first 11 nucleotides in common with mature human miR-155 sequence.

[00280] A further alignment can be made between nucleotides 14 to 18 of the *Ciona savignyi* miR-155 sequence and nucleotides 14 to 18 of the human miR sequence, with conserved sequence regions underlined as depicted:

Homo sapiens miR-155:

5′-UUAAUGCUAAUC—GUGAUAGGGGU-3′ (SEQ ID NO:1)

Ciona savignyi miR-155:

5′-UUAAUGCUAAUAAGUGAUUUAUG-3′ (SEQ ID NO:5)

[00281] Thus is appears that at least 16 of the first 17 nucleotides are evolutionarily conserved between invertebrate (*Ciona savignyi*) and mammalian (*Homo sapiens*) miR-155 sequence, with 20 of the first 21 nucleotides conserved between mature *Ciona intestinalis* and human miR-155 sequences.

[00282] Alignment of mature mouse and human miR-155 transcripts with an evolutionarily conserved C/EBPβ 3′-UTR region that contains a miR-155 target indicates that there is the potential for extensive nucleotide base pairing between miR-155 sequence and the predicted miR-155 target sequence (FIG. 28). Notably, all miR-155 alignments, except those with *Cavia porcellus* (guinea pig) and *Gallus gallus* (chicken) C/EBPβ 3′-UTR sequences, demonstrated perfect matches with the 8-nucleotide miR-155 seed sequence, a major determinant of miRNA binding efficiency. Mismatches with *Cavia porcellus* and *Gallus gallus* C/EBPβ 3′-UTR sequence suggest that miR-155 may not regulate C/EBPβ expression through these 3′-UTR sites, although the potential for alternative base pair formation between the miR-155 seed-sequence and its alignment site in the *Cavia porcellus* C/EBPβ 3′-UTR may be sufficient to permit functional interaction.

[00283] As shown in FIG. 28A, in addition to canonical adenine-to-uracil and cytosine-to-guanine base pairs, the human miR-155 sequence appears capable of forming guanine-to-uracil wobble base pairs with its conserved target site in C/EBPβ 3′-UTR. Specifically, miR-155 nucleotides 22, 20, 19, 14, and 13 may form uracil-to-guanine or guanine-to-uracil wobble base pairs with aligned C/EBPβ 3′-UTR mRNA sequence. Since the thermodynamic stability guanine-to-uracil pairs is similar to those of canonical adenine-to-uracil and cytosine-to-guanine base pairs, the predicted additional sequence complementarity resulting from guanine-to-uracil pairing would be expected to greatly increase miR-155 binding. Moreover, the position of miR-155 nucleotides capable of forming guanine-to-uracil pairs with the human C/EBPβ target site would extend four single nucleotide complementary base pairs at miR-155 nucleotides 23, 21, 18 and 15, into 2 adjacent, unbroken regions of sequence complementarity (miR-155 nucleotides 23-18 and 15-13). Such wobble base pairing would be expected to extend miR-155 specificity and avidity for this site, by increasing the number of miR to mRNA nucleotide base pairs, reducing miRNA nucleotide displacement at canonical nucleotide mismatches to increase base pair hydrogen bonding and miRNA base-stacking energies.

[00284] Based on this sequence alignment data, synthetic miR-155 mimics with improved human C/EBPβ specificity could be created by adenine substitutions at human miR-

155 nucleotides 22, 20, 19, 17, 13, 12, or 11; by a cytosine substitution at miR-155 nucleotide 14; and/or by a guanine substitution at miR-155 nucleotide 10, in order to convert nucleotide mismatches or potential guanine-to-uracil wobble base pairs to canonical adenine-to-uracil and cytosine-to-guanine base pairs (FIG. 28B).

[00285] Adenine substitutions at positions 22, 20, 19 and/or 13 of a synthetic miR-155 sequence should increase the specificity of miR-155 for C/EBPβ by reducing sequence complementarity with related mRNA recognition sequences that have cytosine residues at the relevant mRNA alignment potions. However, adenine substitutions at positions 22, 20, 19 and/or 13 would also be expected to reduce the binding efficiency of miR-155 for C/EBPβ mRNA due to the reduced energetics of adenine *vs.* guanine base stacking. Conversely, a cytosine substitution at positions 14 would be expected to increase both C/EBPβ sequence specificity and avidity due to an increase in sequence complementarity and more favorable cytosine vs. uracil base-stacking energetics at this alignment position. Substitution of adenine positions 12 and/or 11, guanine at position 10, and/or uracil at position 9 of a synthetic miR-155 mimic would also be expected to increase both C/EBPβ sequence specificity and avidity, especially if these substitutions extend an already existing region of sequence complementarity (*i.e.,* nucleotide substitutions at position 12 or 9). Finally, guanine nucleotide substitutions at positions 17, 12, and or 11 could be used to increase base-stacking energy of complementary sequence, although with potential reductions in sequence specificity due to the possibility of increased sequence complementarity with related mRNA recognition sequences that have cytosine residues at the relevant mRNA alignment potions.

6. REFERENCES

[00286] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein in their entirety by express reference thereto:

[00287] U.S. Patent 7,393,478 entitled "Therapy for human cancers using cisplatin and other drugs or genes encapsulated into liposomes."

[00288] U.S. Patent 7,384,923 entitled "Liposomes."

[00289] U.S. Patent 7,371,404 entitled "Amphoteric liposomes and their use."

[00290] U.S. Patent 7,354,567 entitled "Method of encapsulating metal complex within liposomes."

[00291] U.S. Patent 7,312,206 entitled "Sterol derivatives, liposomes comprising sterol derivatives and method for loading liposomes with active substances."

[00292] U.S. Patent 7,273,620 entitled "Triggered release of liposomal drugs following mixing of cationic and anionic liposomes."

[00293] U.S. Patent 7,205,273 entitled "Fusogenic liposomes."

[00294] U.S. Patent 7,153,933 entitled "Solid phase method for synthesis peptide-spacer-lipid conjugates, conjugates synthesized thereby and targeted liposomes containing the same."

[00295] U.S. Patent 7,153,490 entitled "Liposomes encapsulating anticancer drugs and use thereof in the treatment of malignant tumors."

[00296] U.S. Patent 7,150,883 entitled "Self forming, thermodynamically stable liposomes and their applications."

[00297] U.S. Patent 7,067,697 entitled "Cationic liposomes for gene transfer."

[00298] U.S. Patent 6,989,153 entitled "Radiation sensitive liposomes."

[00299] U.S. Patent 6,964,778 entitled "Temperature controlled content release from liposomes."

[00300] U.S. Patent 6,958,160 entitled "Self forming, thermodynamically stable liposomes and their applications."

[00301] U.S. Patent 6,767,554 entitled "Use of complexes among cationic liposomes and polydeoxyribonucleotides and medicaments."

[00302] U.S. Patent 6,743,638 entitled "Detection system using liposomes and signal modification."

[00303] U.S. Patent 6,726,926 entitled "Gene-entrapped liposomes preparation and process for the preparation thereof."

[00304] U.S. Patent 6,610,322 entitled "Self forming, thermodynamically stable liposomes and their applications."

[00305] U.S. Patent 6,610,304 entitled "Liposomes containing multiple branch peptide constructions for use against human immunodeficiency virus."

[00306] U.S. Patent 6,596,543 entitled "Use of liposomes of defined composition and size for the preparation of prothrombin time reagents."

[00307] U.S. Patent 6,596,305 entitled "Method of controlling the size of liposomes."

[00308] U.S. Patent 6,593,294 entitled "Pharmaceutical composition comprising Factor VIII and neutral liposomes."

[00309] U.S. Patent 6,592,843 entitled "Radioactive therapeutic liposomes."

[00310] U.S. Patent 6,511,677 entitled "Polymerizable fatty acids, phospholipids and polymerized liposomes therefrom."

[00311] U.S. Patent 6,511,676 entitled "Therapy for human cancers using cisplatin and other drugs or genes encapsulated into liposomes."

[00312] U.S. Patent 6,492,093 entitled "Radiation-sensitive mixtures comprising IR-absorbing cyanine dyes having a betaine structure or having a betaine structure and containing an anion, and recording materials prepared therewith."

[00313] U.S. Patent 6,469,084 entitled "Process for preparing an aqueous composition in gel form and compositions obtainable from this process, especially a composition containing vesicles, in particular liposomes."

[00314] U.S. Patent 6,458,381 entitled "Lipids and their use, for example, in liposomes."

[00315] U.S. Patent 6,451,338 entitled "Liposomes containing particulate materials."

[00316] U.S. Patent 6,426,086 entitled "pH-sensitive, serum-stable liposomes."

[00317] U.S. Patent 6,387,397 entitled "Polymerized liposomes targeted to M cells and useful for oral or mucosal drug delivery."

[00318] U.S. Patent 6,380,359 entitled "Liposomes comprising peptide antigens derived from X protein of hepatitis B virus."

[00319] U.S. Patent 5,540,936 entitled "Method of producing liposomes."

[00320] PCT Intl. Pat. Appl. Publ. No. WO95/08348.

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[00398] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of exemplary embodiments, it will be apparent to those of ordinary skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically- and physiologically-related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those of ordinary skill in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. Accordingly, the exclusive rights sought to be patented are as described in the claims below.

THE CLAIMS:

1. A method of modulating the expression of one or more genes associated with a target cell involved in mammalian adipogenesis, which comprises contacting the target cell with an miR-155 composition in an amount and for a time sufficient to modulate the expression of the one or more genes.

2. The method of claim 1, wherein the miR-155 composition comprises an miR-155 agonist or antagonist.

3. The method of claim 2, wherein the miR-155 agonist or antagonist comprises an antisense or siRNA molecule.

4. The method of claim 3, wherein the miR-155 composition comprises an oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 80% identical to native microR-155, precursor-microRNA-15, or any one of SEQ ID NO:1-SEQ ID NO:5; or (b) is complementary to a sequence at least 80% identical to mature microRNA-155, pre-microR-155, or any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

5. The method of claim 4, wherein the miR-155 composition comprises an oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 85% identical to any one of SEQ ID NO:1 to SEQ ID NO:5; or (b) is complementary to a sequence at least 85% identical to any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

6. The method of claim 5, wherein the miR-155 composition comprises an oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 90% identical to any one of SEQ ID NO:1 to SEQ ID NO:5; or (b) is complementary to a sequence at least 90% identical to any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

7. The method of claim 6, wherein the miR-155 composition comprises an oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 95% identical to any one of SEQ ID NO:1 to SEQ ID NO:5; or (b) is complementary to a sequence at least 95% identical to any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

8. The method of claim 1, wherein the modulation comprises increasing expression of the one or more genes as compared to that expressed in a wild type.

9. The method of claim 1, wherein the modulation comprises decreasing expression of the one or more genes as compared to wild type.

10. A method of treating, preventing, or ameliorating one or more symptoms of an miR-155 associated disease or condition selected from the group consisting of diabetes, obesity, overeating, excessive body weight gain, and hyperlipidemia in a mammal, the method comprising:

(a) identifying a mammalian subject having, or suspected of having an miR-155 associated disease or condition; and

(b) administering to the mammalian subject a sufficient amount of a miR-155 composition, analog, agonist, or antagonist thereof for a time sufficient to treat, prevent, or ameliorate the one or more symptoms of the miR-155 associated disease or condition.

11. The method of claim 10, wherein the miR-155 associated condition is an adipogenesis-related condition or an adipogenic disorder or dysfunction.

12. The method of claim 10, wherein the miR-155 composition comprises a miR-155 agonist or antagonist.

13. The method of claim 12, wherein the miR-155 antagonist comprises an miR-155 antisense or siRNA compound.

14. The method of claim 13, wherein the miR-155 antisense compound comprises a modified oligonucleotide consisting of 12 to 30 linked nucleosides, wherein the nucleobase sequence of the modified oligonucleotide is complementary to a sequence at least 80% identical to native miR-155, precursor-mR-155, or any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5; or an analog or any combination thereof.

15. The method of claim 14, wherein the nucleobase sequence of the miR-155 oligonucleotide has no more than two mismatches to the nucleobase sequence of native miR-155, or precursor-miR-155.

16. The method of claim 15, wherein the nucleobase sequence of the miR-155 oligonucleotide has no more than three mismatches to the nucleobase sequence of native miR-155, or precursor-miR-155.

17. The method of claim 16, wherein the nucleobase sequence of the miR-155 oligonucleotide has no more than four mismatches to the nucleobase sequence of native miR-155, or precursor-miR-155.

18. The method of claim 10, wherein the mammalian subject is human.

19. A method for providing a therapeutic amount of an miR-155 composition to a first cell in an animal, comprising providing to an animal in need thereof a therapeutically-effective amount of an miR-155 oligonucleotide for a time sufficient to treat or ameliorate one or more symptoms of a condition resulting from expression of at least a first gene expressed in at least a first adipogenic or antiadipogenic pathway.

20. The method of claim 19, wherein the miR-155 composition is administered systemically to the animal.

21. The method of claim 19, wherein the miR-155 composition is administered: (a) locally to a region of the body including the population of cells or a first tissue site comprising

the population of cells; or (b) *ex vivo* to a population of cells or a first tissue site obtained from at least a first region of the body.

22. The method of claim 18, wherein the animal is human.

23. An miR-155 composition for use in the therapy of diabetes, obesity, overeating, excessive body weight gain, excessive fat accumulation, or hyperlipidemia in a mammal, wherein the composition comprises at least a first oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 80% identical to native microR-155, precursor-microRNA-15, or any one of SEQ ID NO:1-SEQ ID NO:5; or (b) is complementary to a sequence at least 80% identical to mature microRNA-155, pre-microR-155, or any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

24. Use of an miR-155 composition that comprises at least a first oligonucleotide that consists of about 12 to about 30 contiguous nucleotides, and (a) is at least 80% identical to native microR-155, precursor-microRNA-15, or any one of SEQ ID NO:1-SEQ ID NO:5; or (b) is complementary to a sequence at least 80% identical to mature microRNA-155, pre-microR-155, or any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5 in the manufacture of a medicament for treating, preventing, or ameliorating one or more symptoms of diabetes, obesity, overeating, excessive body weight gain, excessive fat accumulation, or hyperlipidemia in a mammal.

25. Use in accordance with claim 25, wherein the mammal is human.

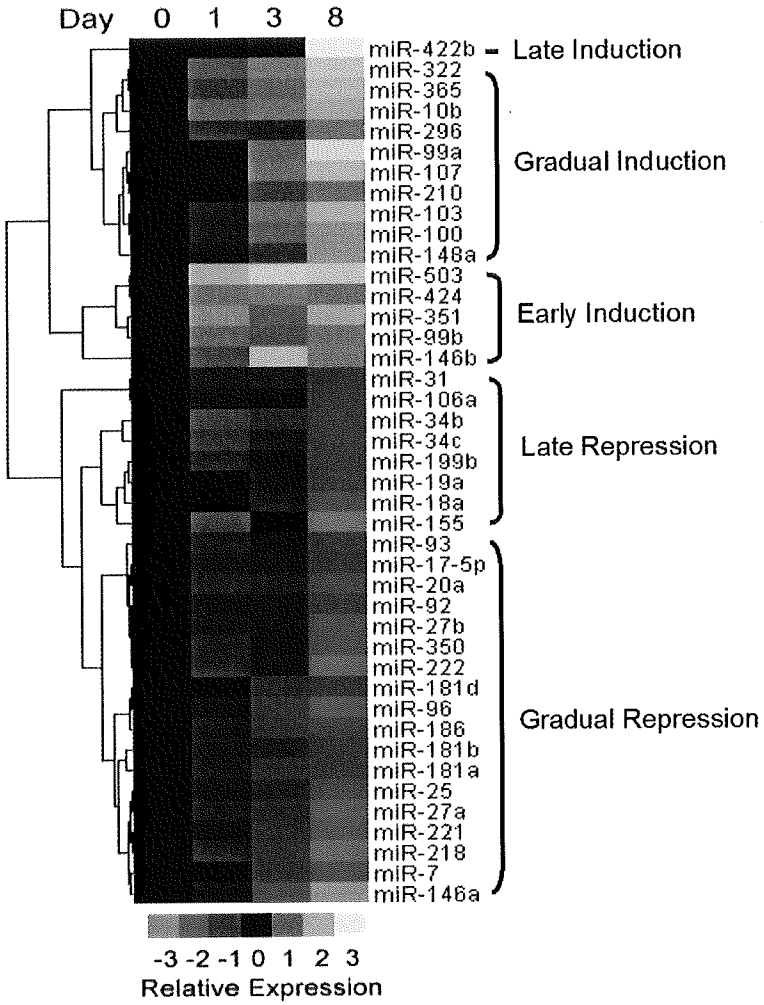
Day 0 1 3 8
miR-422b
miR-322
miR-365
miR-10b
miR-296
miR-99a
miR-107
miR-210
miR-103
miR-100
miR-148a
miR-503
miR-424
miR-351
miR-99b
miR-146b
miR-31
miR-106a
miR-34b
miR-34c
miR-199b
miR-19a
miR-18a
miR-155
miR-93
miR-17-5p
miR-20a
miR-92
miR-27b
miR-350
miR-222
miR-181d
miR-96
miR-186
miR-181b
miR-181a
miR-25
miR-27a
miR-221
miR-218
miR-7
miR-146a
Late Induction
Gradual Induction
Early Induction
Late Repression
Gradual Repression
-3 -2 -1 0 1 2 3
Relative Expression

FIG. 1

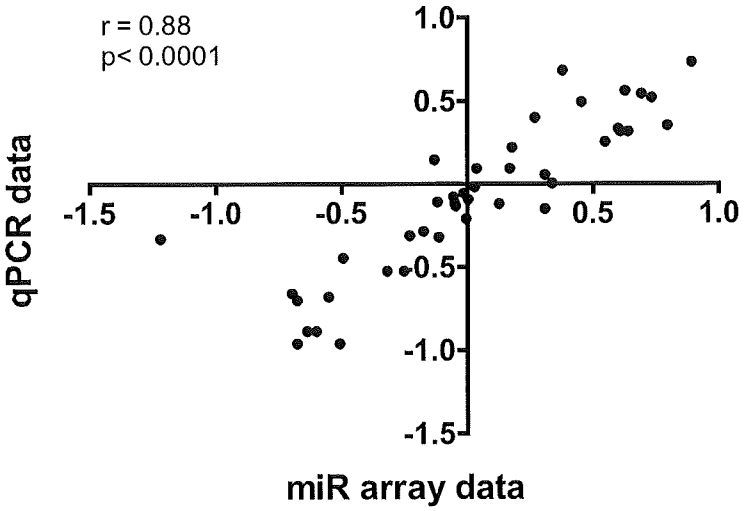
r = 0.88
p< 0.0001
qPCR data
miR array data
1.0
0.5
-0.5
-1.0
-1.5
-1.5
-1.0
-0.5
0.5
1.0

FIG. 2

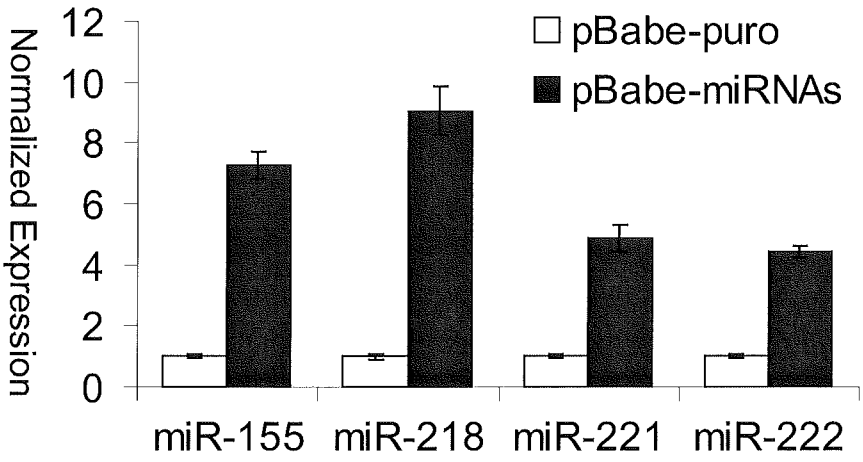
Normalized Expression
12
10
8
6
4
2
0
pBabe-puro
pBabe-miRNAs
miR-155
miR-218
miR-221
miR-222

FIG. 3A

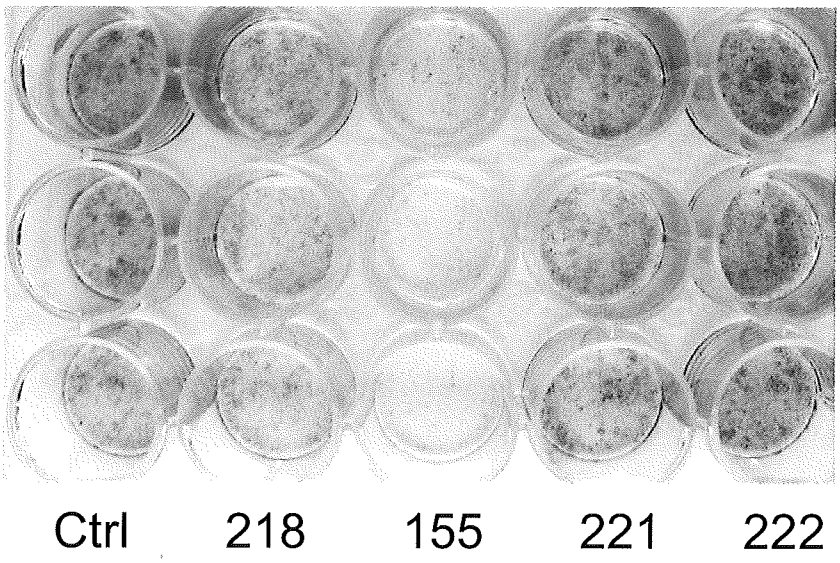
Ctrl
218
155
221
222

FIG. 3B

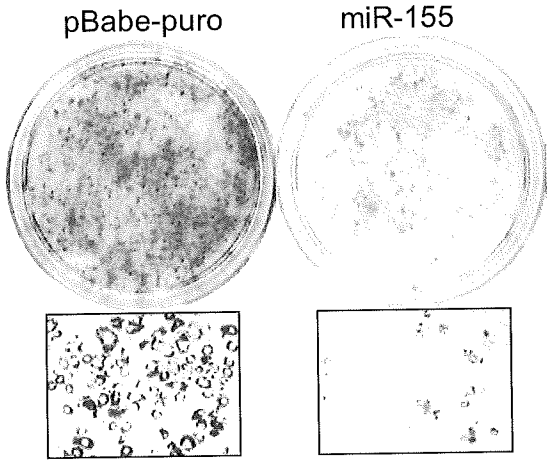

FIG. 4A

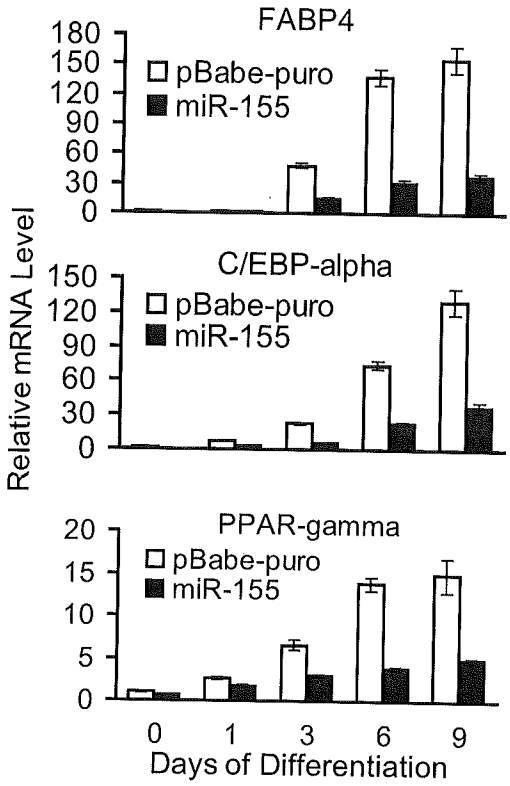

FIG. 4B

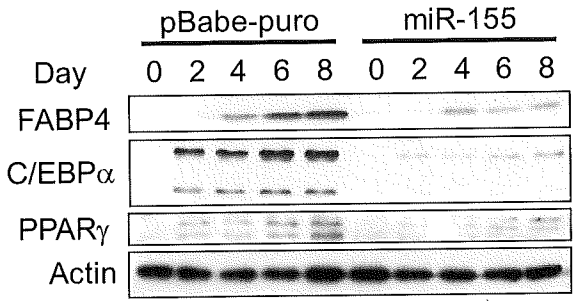

FIG. 4C

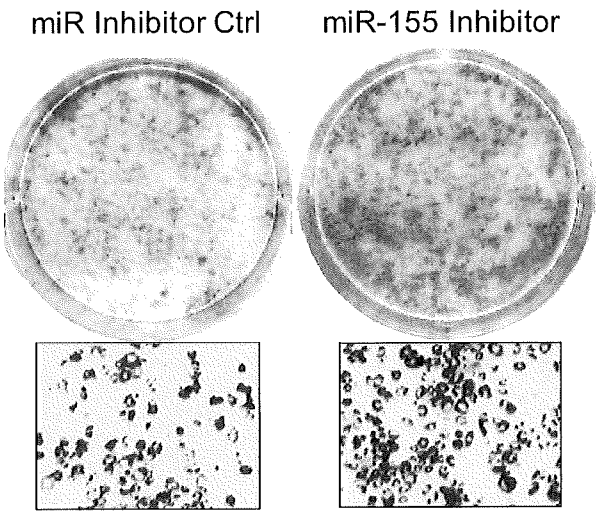
miR Inhibitor Ctrl
miR-155 Inhibitor

FIG. 5A

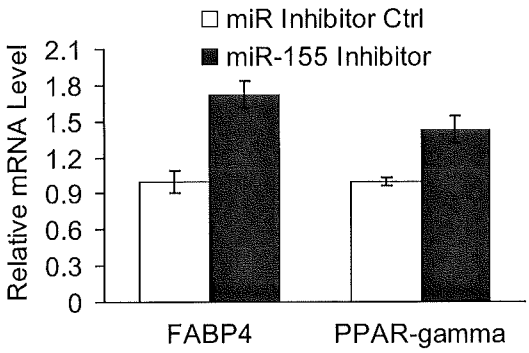
miR Inhibitor Ctrl
miR-155 Inhibitor
Relative mRNA Level
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
FABP4
PPAR-gamma

FIG. 5B

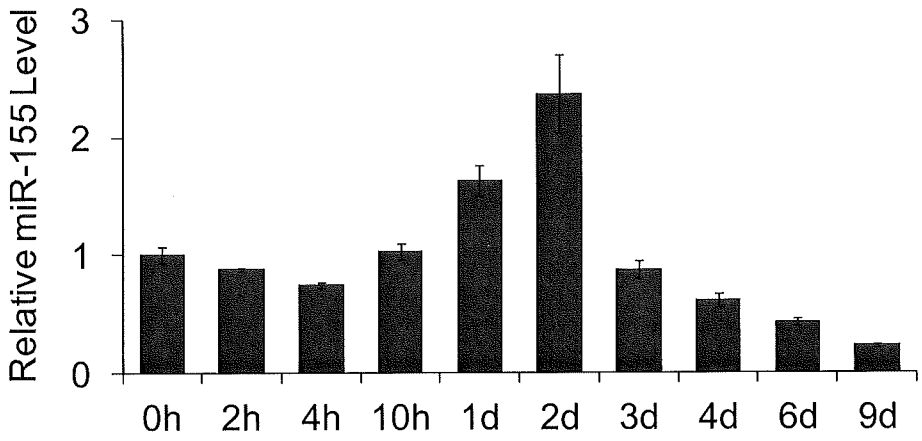

FIG. 6

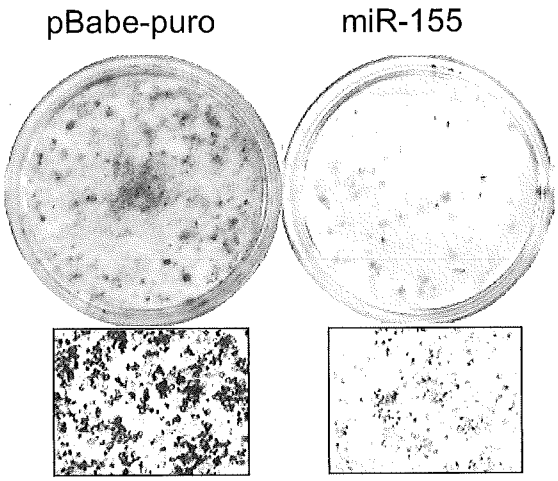
pBabe-puro
miR-155

FIG. 7A

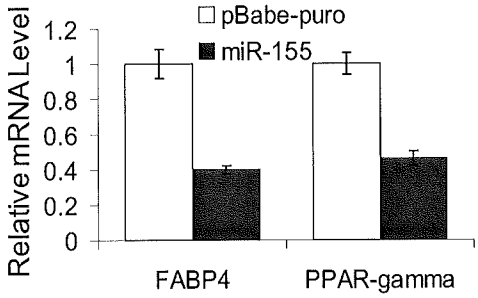
pBabe-puro
miR-155
Relative mRNA Level
1.2
1
0.8
0.6
0.4
0.2
0
FABP4
PPAR-gamma

FIG. 7B

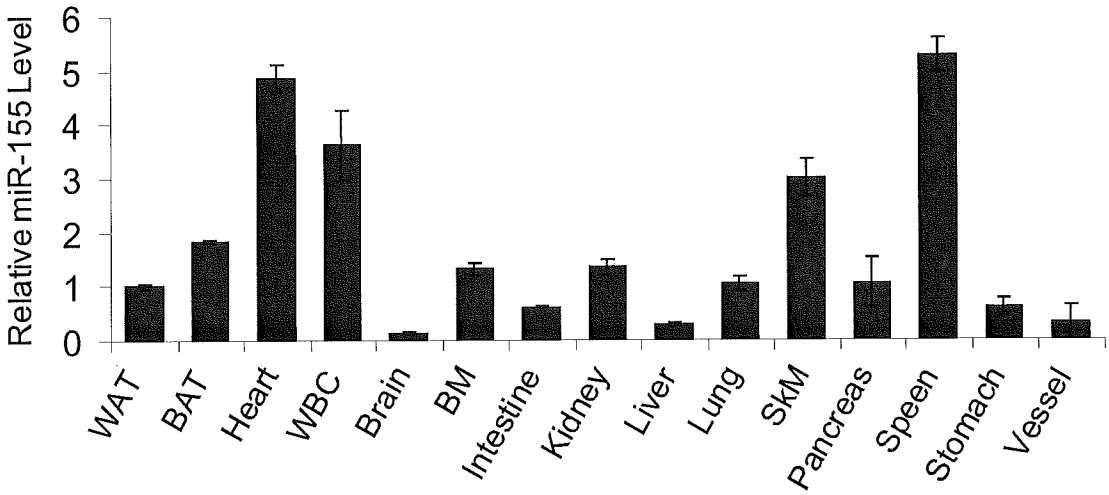
Relative miR-155 Level
0
1
2
3
4
5
6
WAT
BAT
Heart
WBC
Brain
BM
Intestine
Kidney
Liver
Lung
SkM
Pancreas
Speen
Stomach
Vessel

FIG. 8A

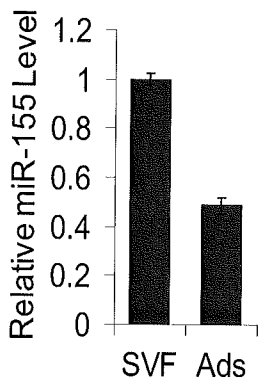
Relative miR-155 Level
0
0.2
0.4
0.6
0.8
1
1.2
SVF
Ads

FIG. 8B

mmu-miR-155 3' UGGGGAUAGUGUUAAUCGUAAUU 5' (SEQ ID NO:15)
C/EBPβ 3'-UTR
Mouse 5'---A CCUUUUUCCGUUUUCGAGCAUUAA---3' (SEQ ID NO:16)
Human 5'---A UCUUUUUCCGUUUUCAAGCAUUAA---3' (SEQ ID NO:17)
Rat 5'---A CCUUUUUCCGUUUUCGAGCAUUAA---3' (SEQ ID NO:18)
Dog 5'---A UCUUUUUCCGUUUUCAAGCAUUAA---3' (SEQ ID NO:19)
Chicken 5'---U CUUGCUACAUUUUGAAGCAUUAA---3' (SEQ ID NO:20)

FIG. 9

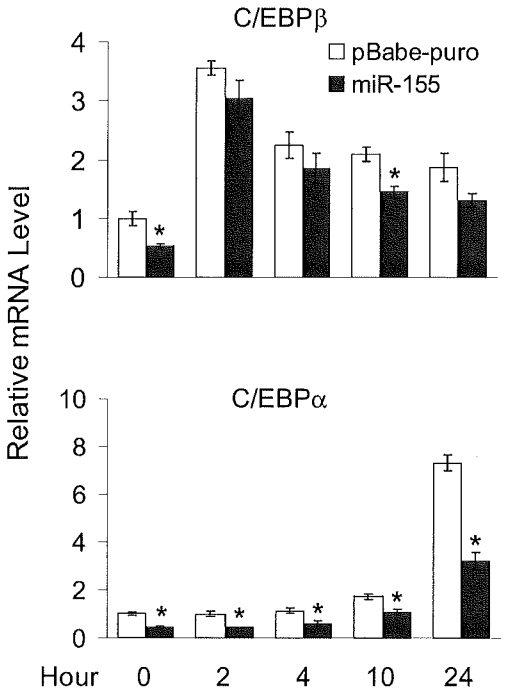

FIG. 10A

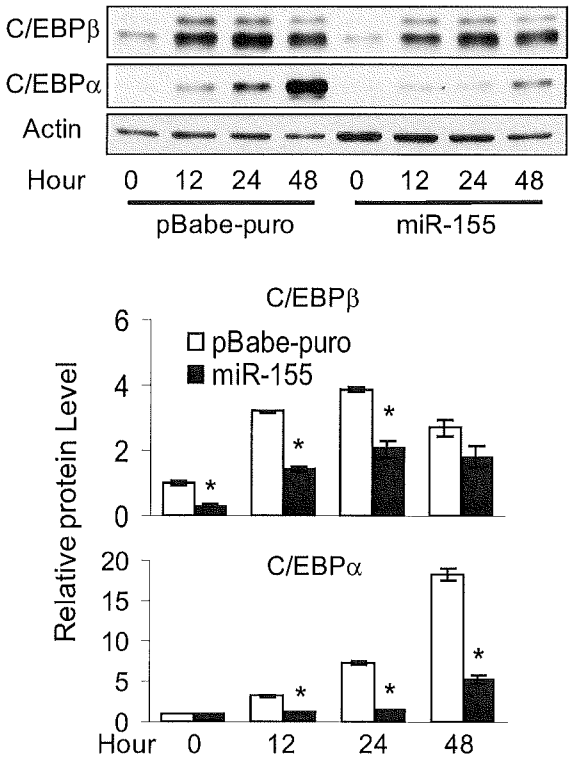

FIG. 10B

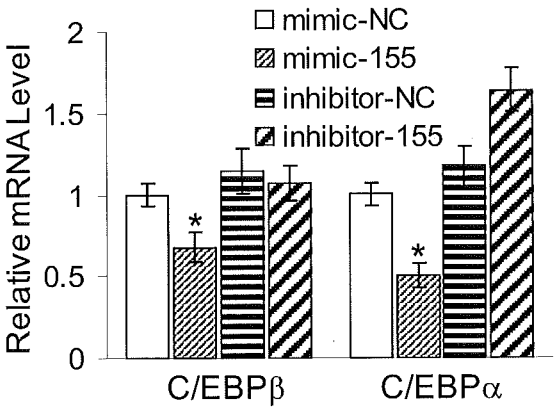

FIG. 11A

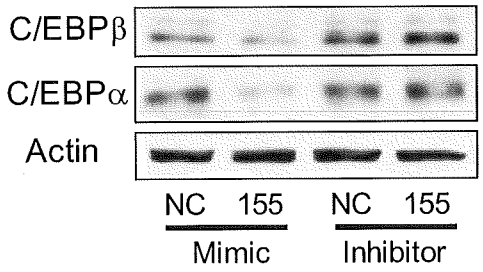

FIG. 11B

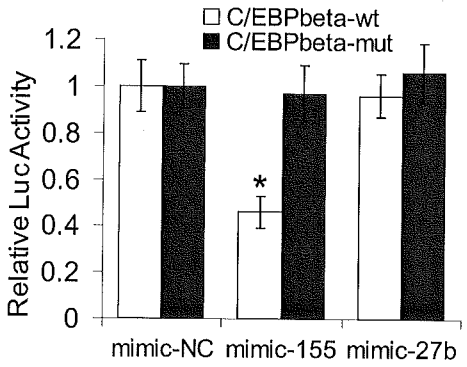

FIG. 11C

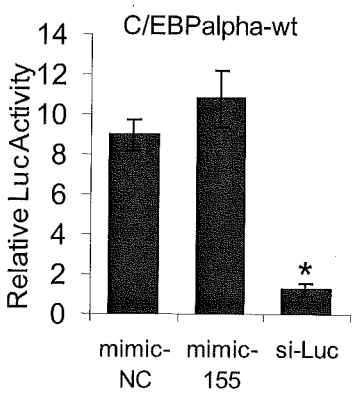

FIG. 11D

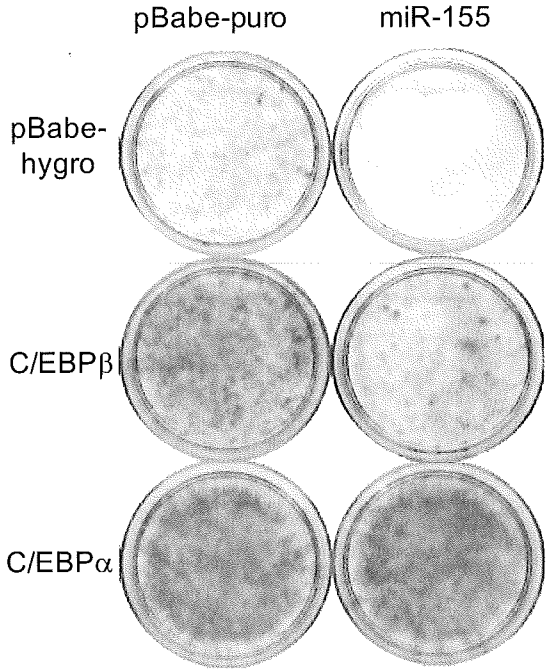

FIG. 12A

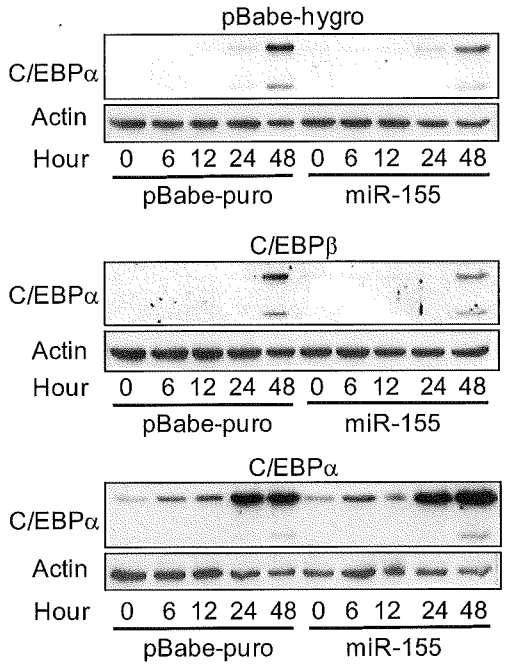

FIG. 12B

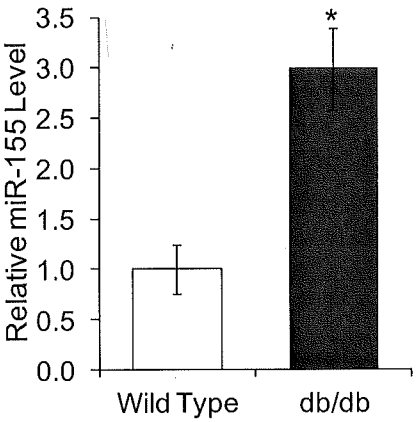

FIG. 13A

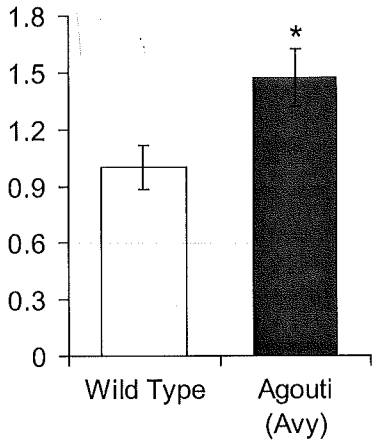

FIG. 13B

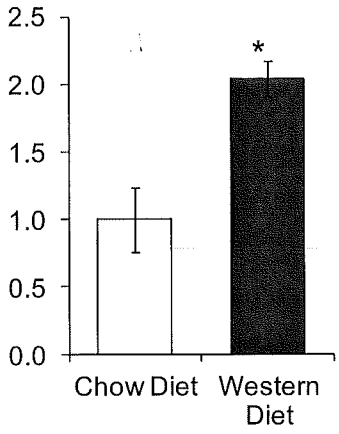

FIG. 13C

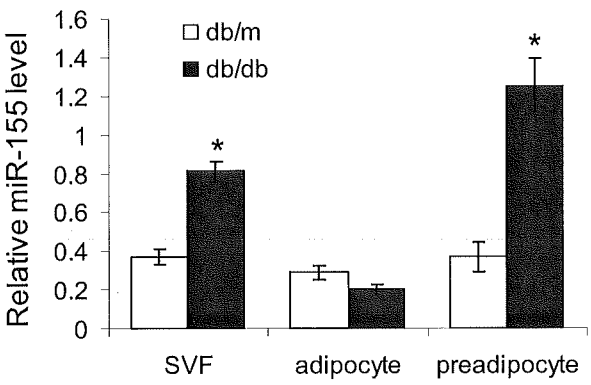

FIG. 14A

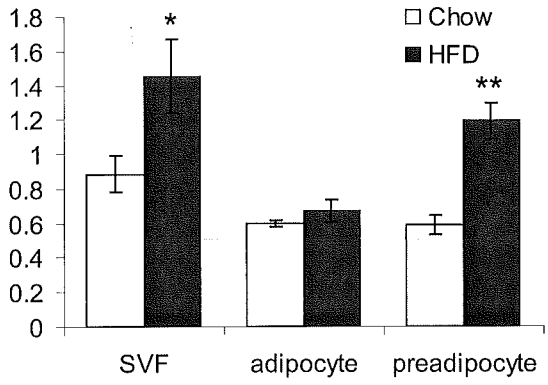

FIG. 14B

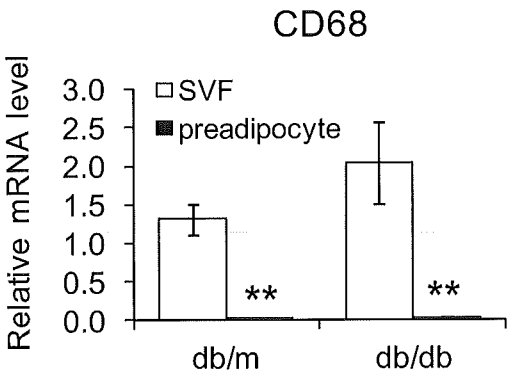

FIG. 15A

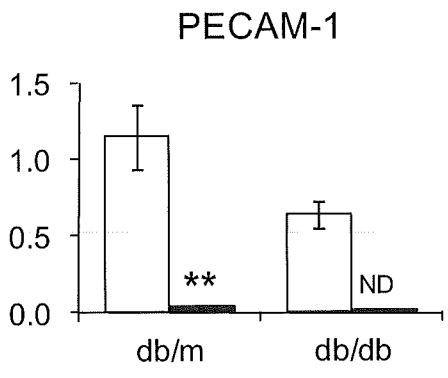

FIG. 15B

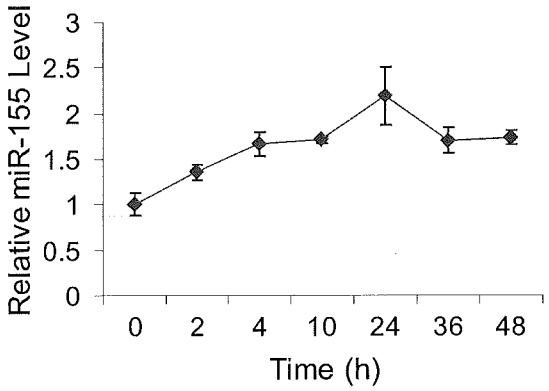

FIG. 16A

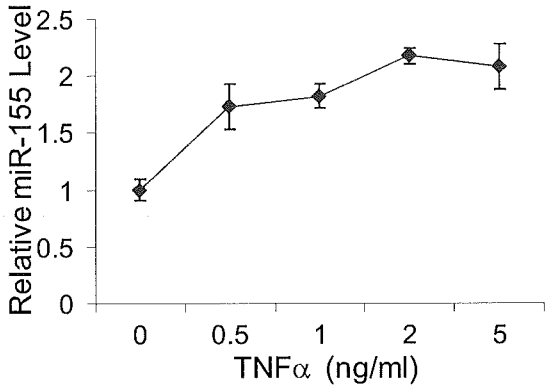

FIG. 16B

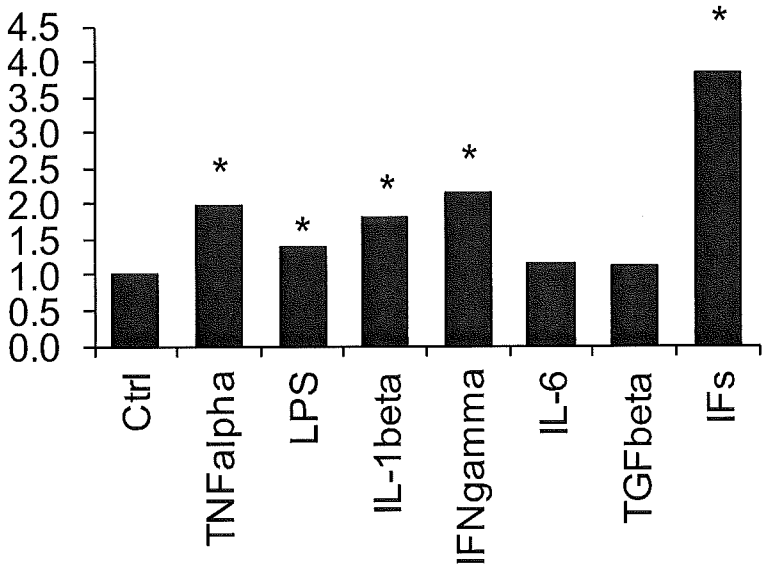
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
Ctrl
TNFalpha
LPS
IL-1beta
IFNgamma
IL-6
TGFbeta
IFs

FIG. 17

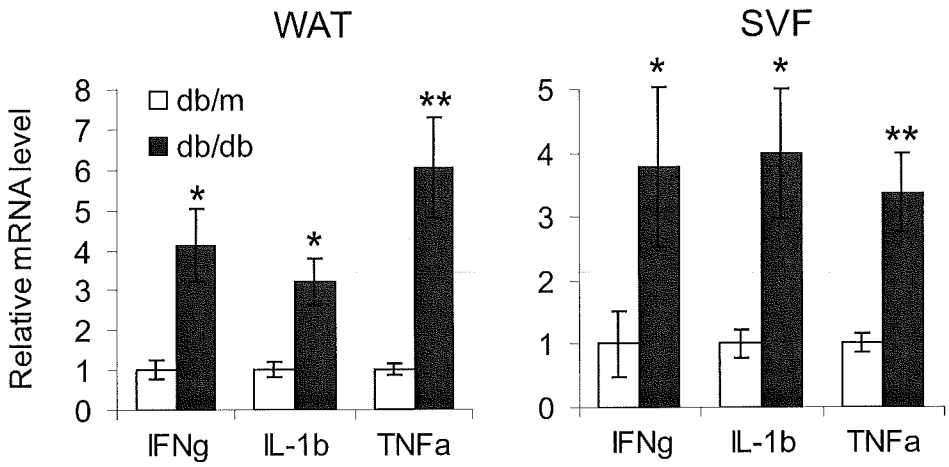

FIG. 18A

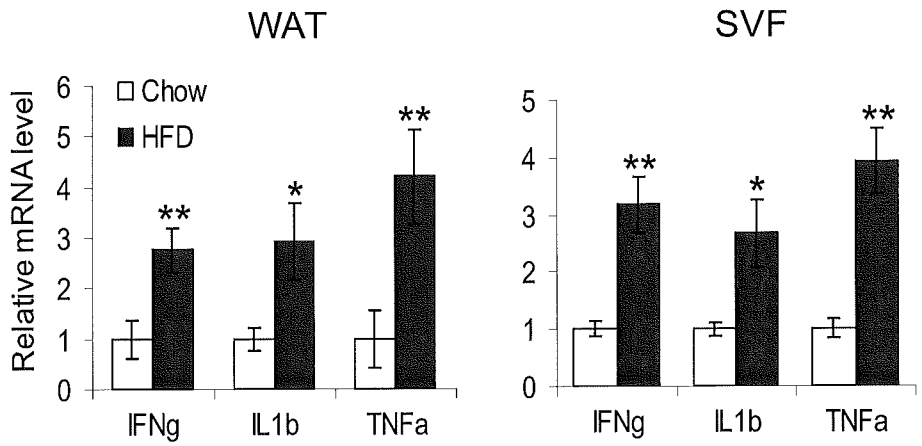

FIG. 18B

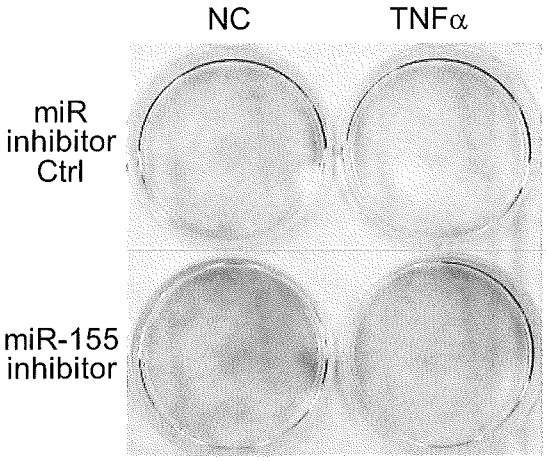

FIG. 19A

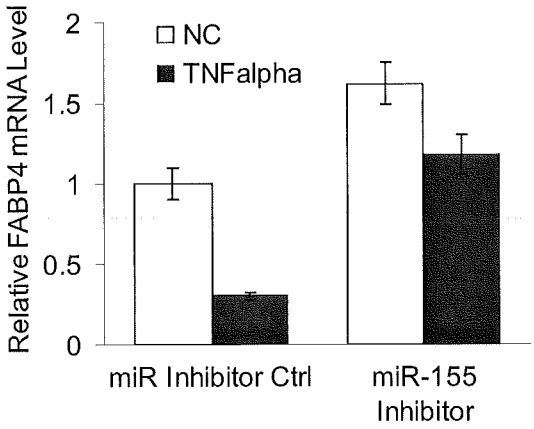

FIG. 19B

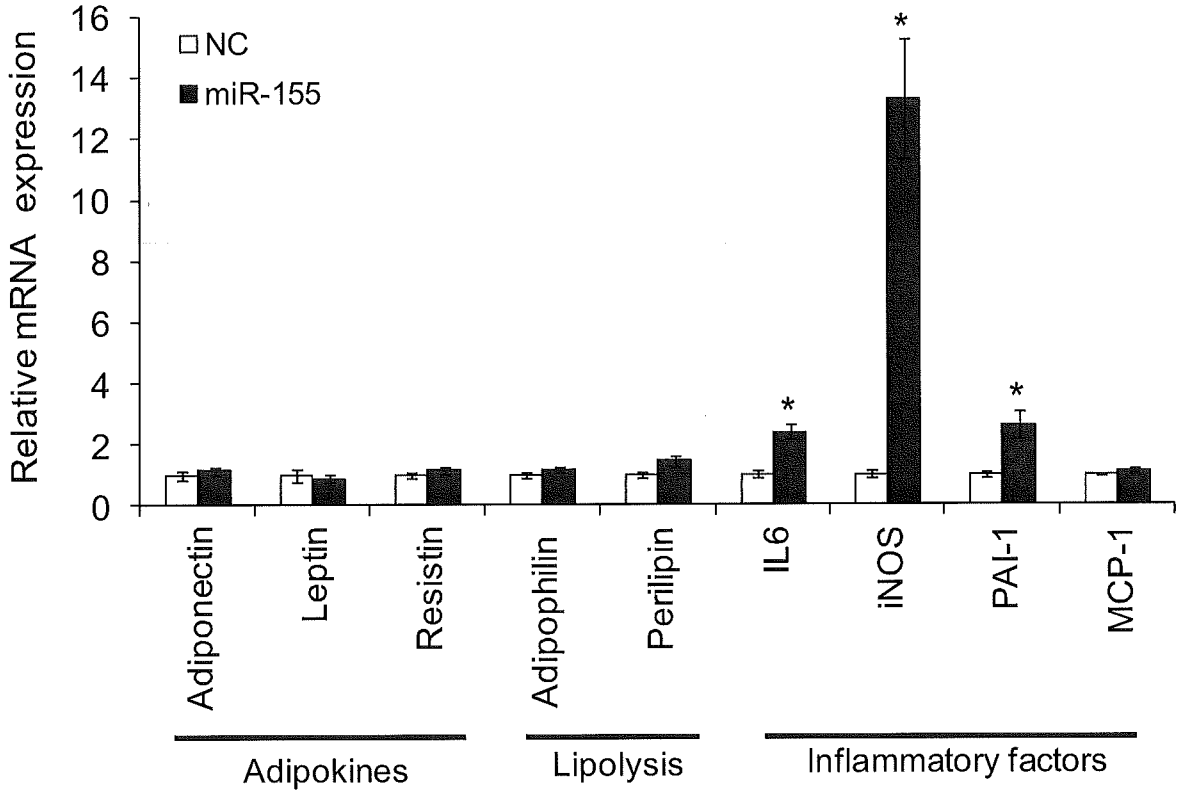
Relative mRNA expression
16
14
12
10
8
6
4
2
0
NC
miR-155
*
*
*
Adiponectin
Leptin
Resistin
Adipophilin
Perilipin
IL6
iNOS
PAI-1
MCP-1
Adipokines
Lipolysis
Inflammatory factors

FIG. 20

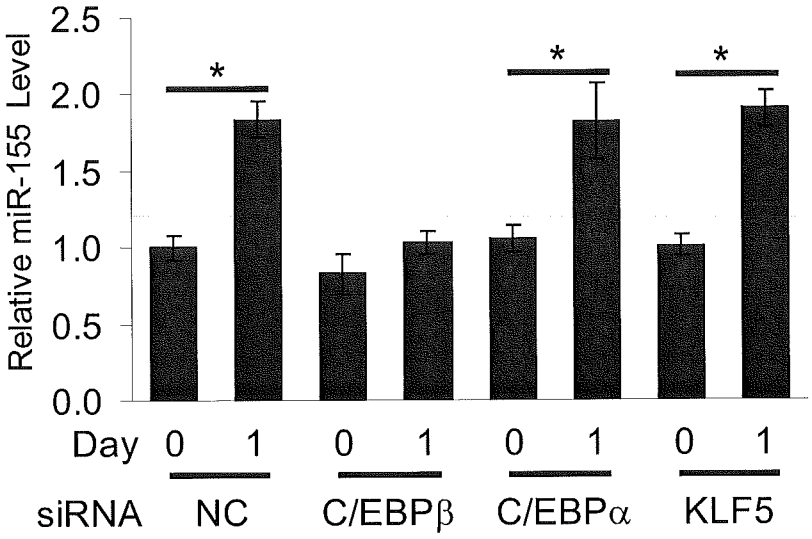

FIG. 21A

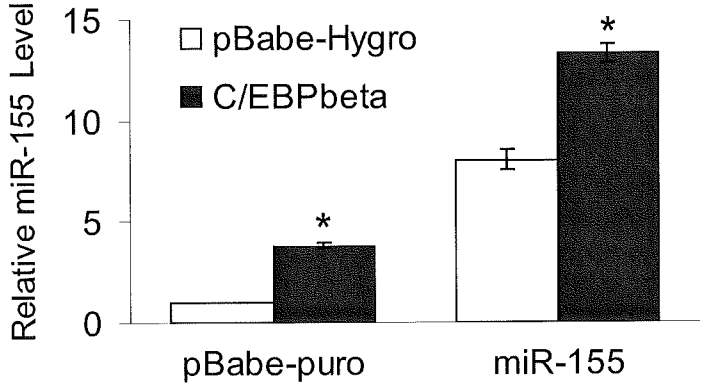

FIG. 21B

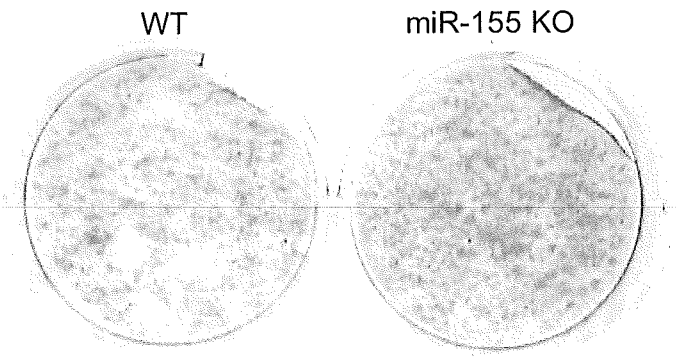

FIG. 22A

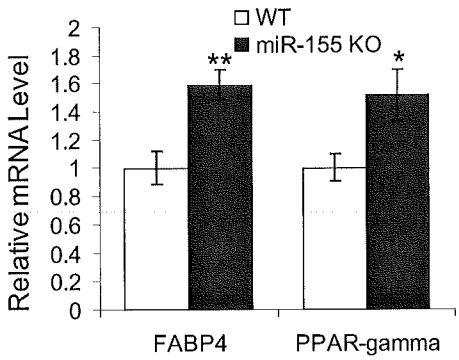

FIG. 22B

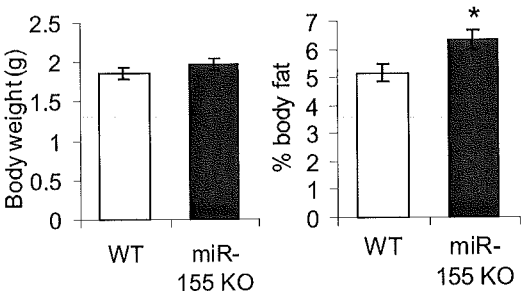

FIG. 23A

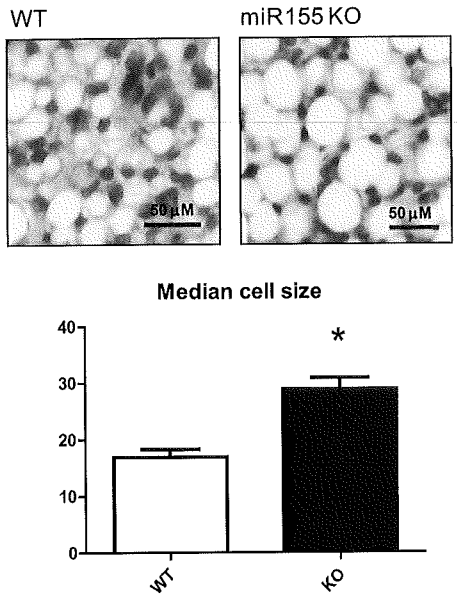

FIG. 23B

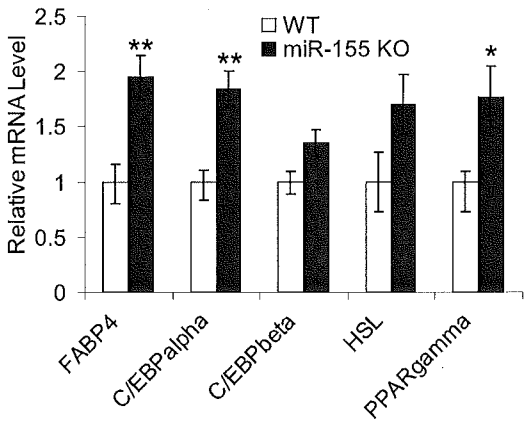

FIG. 23C

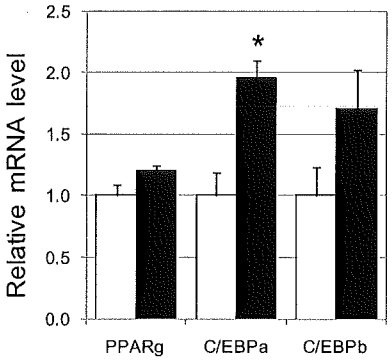

FIG. 24A

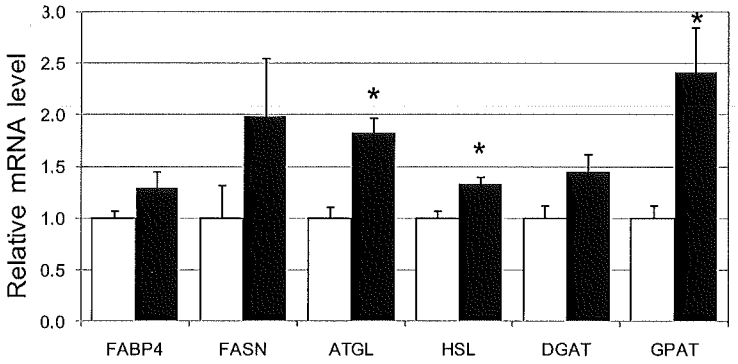

FIG. 24B

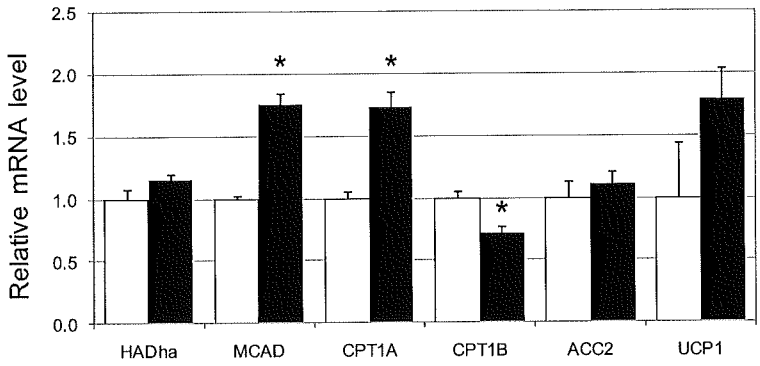

FIG. 24C

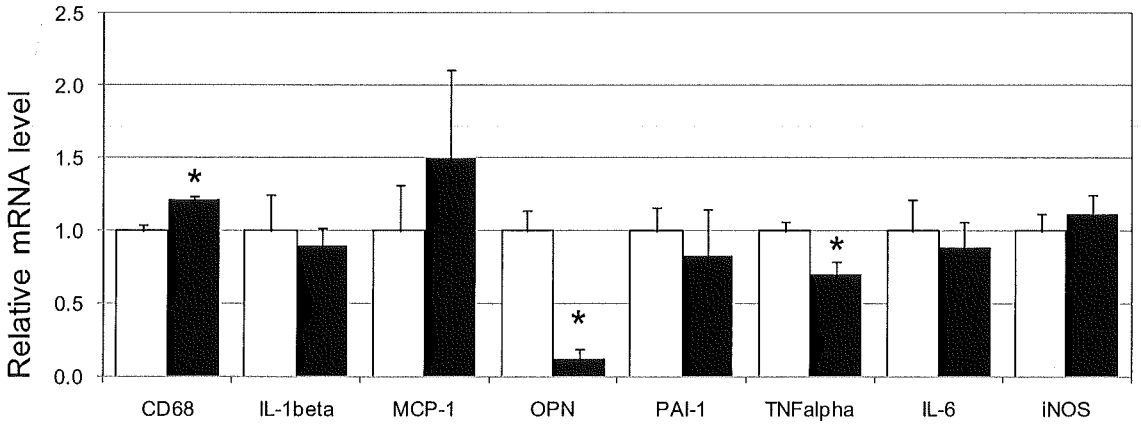

FIG. 25A

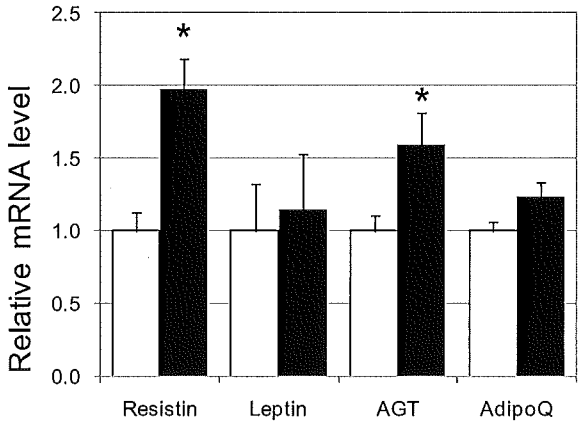

FIG. 25B

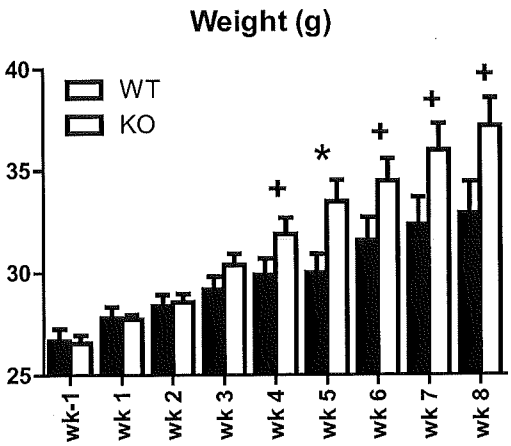

FIG. 26A

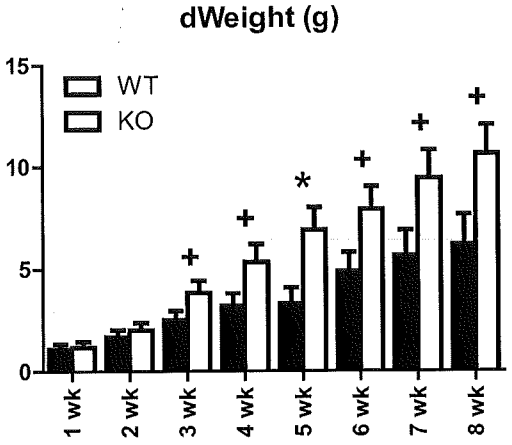

FIG. 26B

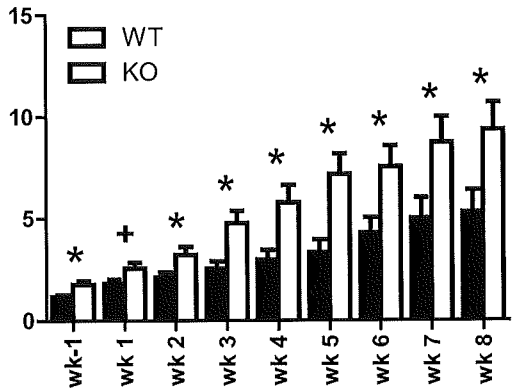

FIG. 26C

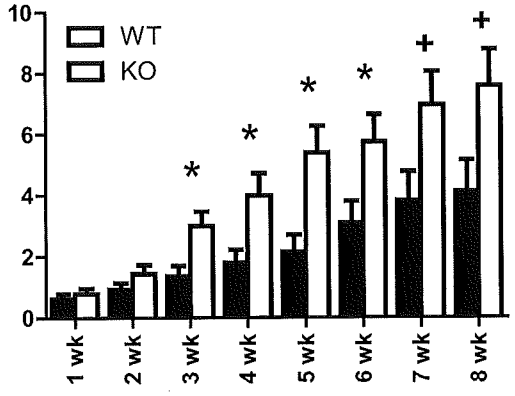

FIG. 26D

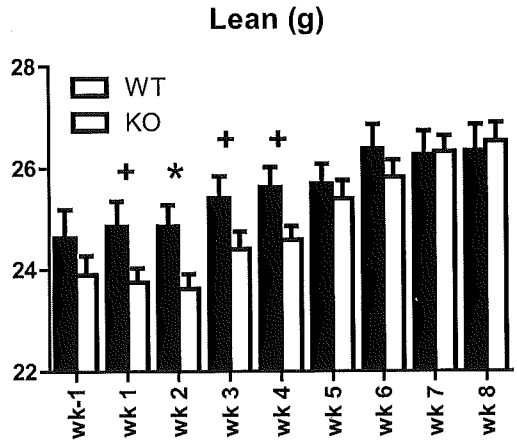

FIG. 26E

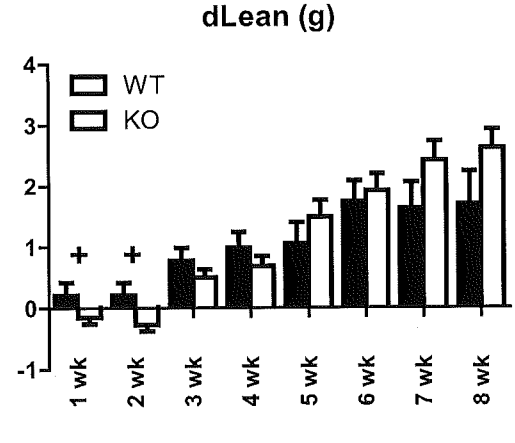

FIG. 26F

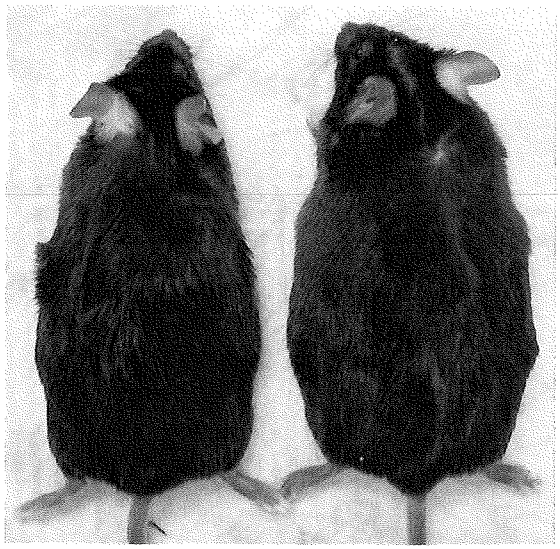

FIG. 27A

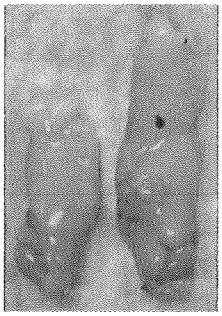

FIG. 27B

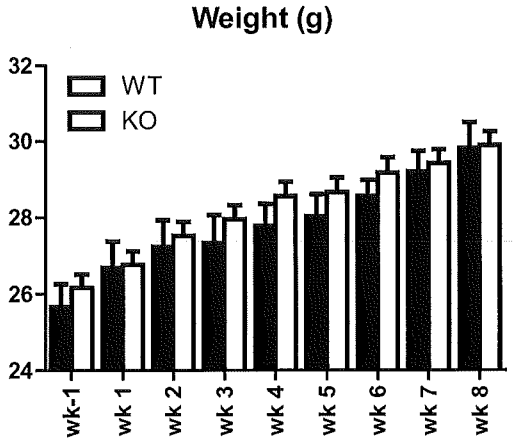

FIG. 28A

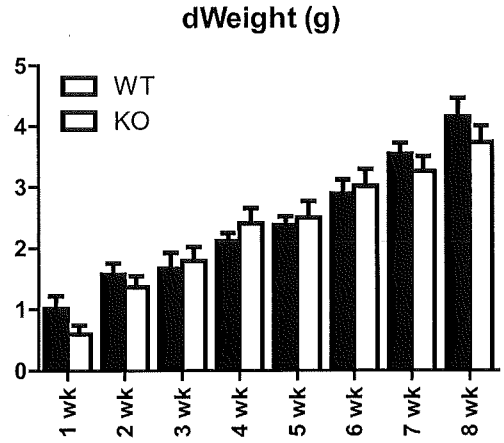

FIG. 28B

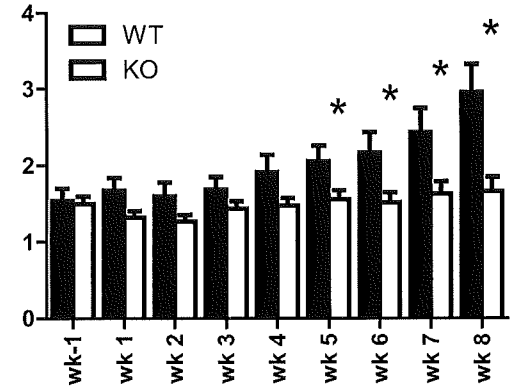

FIG. 28C

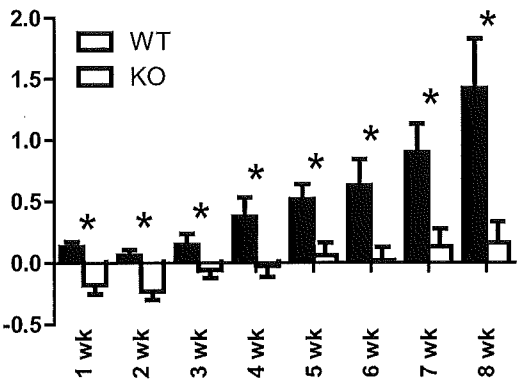

FIG. 28D

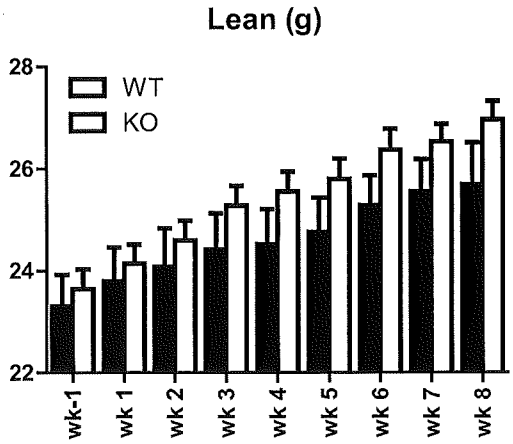

FIG. 28E

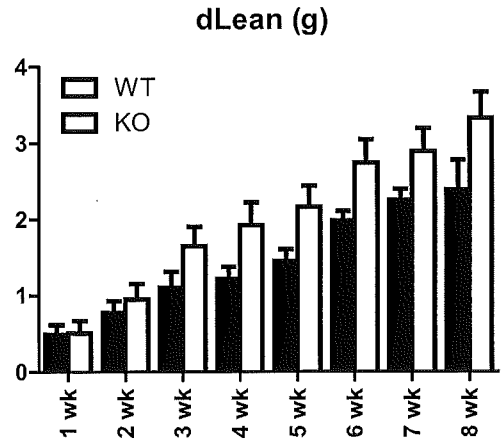

FIG. 28F

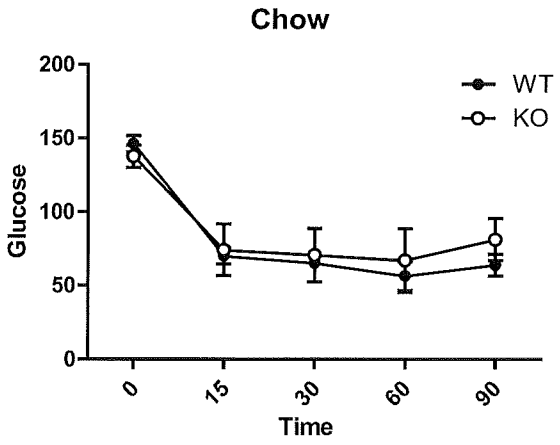

FIG. 29A

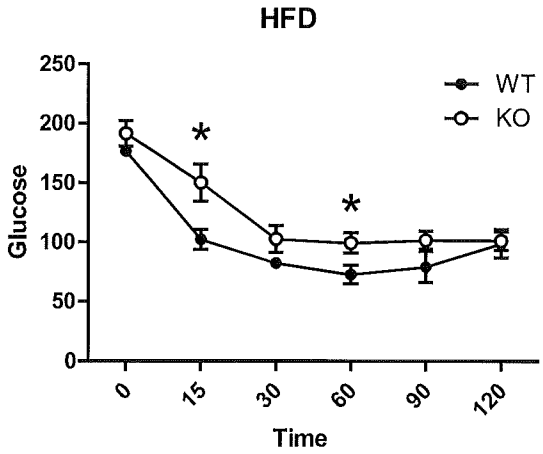

FIG. 29B

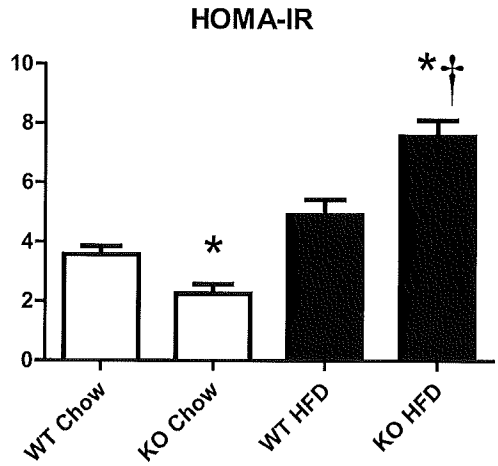

FIG. 29C

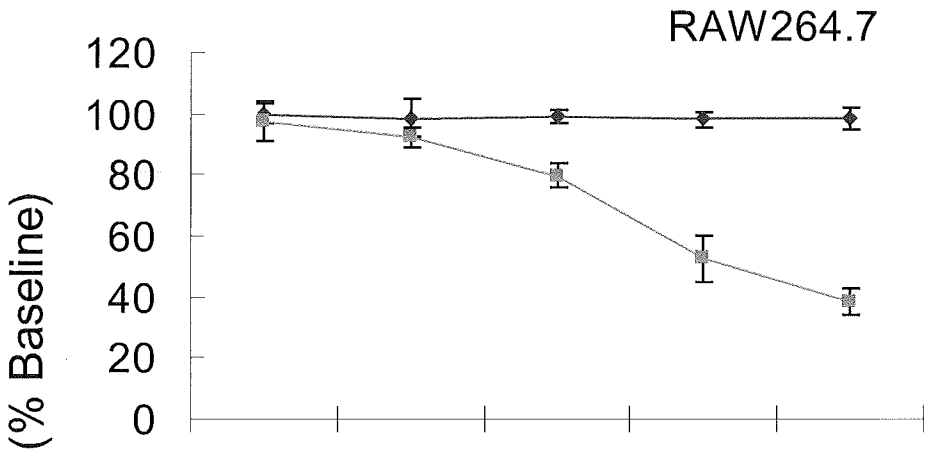

FIG. 30A

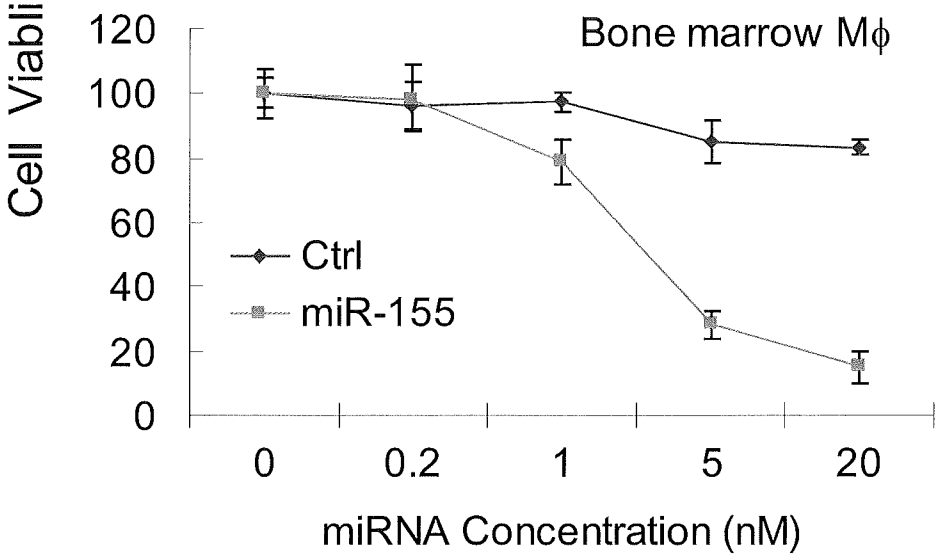

FIG. 30B

Mouse mir-155	3′-UGGGGAUAGUGCUAAUCGUAAUU-5′		(SEQ ID NO:15)
Human mir-155	3′-UGGGGAUAGUGUUAAUCGUAAUU-5′		(SEQ ID NO:21)
C/EBPβ 3′UTR:			
Human	AUCUUUUCCGUUUCAAGCAUUAA	*Homo sapiens*	(SEQ ID NO:22)
Chimp	AUCUUUUCCGUUUCAAGCAUUAA	*Pan troglodytes*	(SEQ ID NO:23)
Dog	AUCUUUUCCGUUUCAAGCAUUAA	*Canis familiaris*	(SEQ ID NO:24)
Horse	AUCUUUUCCGUUUCAAGCAUUAA	*Equus caballus*	(SEQ ID NO:25)
Tenrec	AUCUUUUCCGUUUCAAGCAUUAA	*Echinops telfairi*	(SEQ ID NO:26)
Elephant	AUCUUUUCCGUUUUAAGCAUUAA	*Loxodonta Africana*	(SEQ ID NO:27)
Mouse	ACCUUUUCCGUUUCAAGCAUUAA	*Mus musculus*	(SEQ ID NO:28)
Rat	ACCUUUUCCGUUUCAAGCAUUAA	*Rattus norvegicus*	(SEQ ID NO:29)
Rhesus	AUCUU--CCGUUUCAAGCAUUAA	*Macaca mulatta*	(SEQ ID NO:30)
Opossum	AAUCUCUGCGUUUCAAGCAUUAA	*Monodelphis domestica*	(SEQ ID NO:31)
Rabbit	UCUUUUUCCCUUUCGAGCAUUAA	*Oryctolagus cuniculus*	(SEQ ID NO:32)
Guinea Pig	AAUUCUCCGUCUCCAGGCUUUAG	*Cavia porcellus*	(SEQ ID NO:33)
Chicken	UCUAUGCAUUCUUGCUACAUUUG	*Gallus gallus*	(SEQ ID NO:34)

FIG. 31A

C/EBPβ 3′UTR	5′-AUCUUUUCCGUUUCAAGCAUUAA-3′	
miR-155	3′-UGGGGAUAGUGUUAAUCGUAAUU-5′	(SEQ ID NO:15)
Watson-Crick	-A-AA-A--CAAAGU--------	(SEQ ID NO:35)
Wobble	------G----GG----------	(SEQ ID NO:36)
	22221111111111	
	32109876543210987654321	

FIG. 31B

Homo sapiens	Human	UUAAUGCUAAUCGUGAUAGGGGU	(SEQ ID NO:1
Pan troglodytes	Chimpanzee	UUAAUGCUAAUCGUGAUAGGGGU	
Pongo pygmaeus	Bornean Orangutan	UUAAUGCUAAUCGUGAUAGGGGU	
Macaca mulatta	Rhesus Macaque	UUAAUGCUAAUCGUGAUAGGGGU	
Canis familiaris	Dog	UUAAUGCUAAUCGUGAUAGGGGU	
Bos taurus	Cow	UUAAUGCUAAUCGUGAUAGGGGU	
Equus caballus	Horse	UUAAUGCUAAUCGUGAUAGGGGU	
Mus musculus	Mouse	UUAAUGCUAAUUGUGAUAGGGGU	(SEQ ID NO:2)
Ornithorhynchus anatinus	Platypus	UUAAUGCUAAUCGUGAUAGGGGU	(SEQ ID NO:1)
Gallus gallus	Chicken	UUAAUGCUAAUCGUGAUAGGGG	(SEQ ID NO:3)
Taeniopygia guttata	Zebra finch	UUAAUGCUAAUCGUGAUAGGGG	
Danio rerio	Zebrafish	UUAAUGCUAAUCGUGAUAGGGG	
Xenopus tropicalis	Western clawed frog	UUAAUGCUAAUCGUGAUAGGGG	
Ciona intestinalis	Sea squirt	UUAAUGCUAAUAGUGAUAGGG	(SEQ ID NO:4)
Ciona savignyi	Pacific sea squirt	UUAAUGCUAAUAAGUGAUUUAUG	(SEQ ID NO:5)

FIG. 32A

Homo sapiens	Human	UUAAUGCUAAUCGUGAUAGGGGU (SEQ ID NO:1)
Pan troglodytes	Chimpanzee	----------
Pongo pygmaeus	Bornean Orangutan	----------
Macaca mulatta	Rhesus Macaque	----------
Canis familiaris	Dog	----------
Bos taurus	Cow	----------
Equus caballus	Horse	----------
Mus musculus	Mouse	----------U---------- (SEQ ID NO:2)
Ornithorhynchus anatinus	Platypus	---------- (SEQ ID NO:1)
Gallus gallus	Chicken	---------- (SEQ ID NO:3)
Taeniopygia guttata	Zebra finch	----------
Danio rerio	Zebrafish	----------
Xenopus tropicalis	Western clawed frog	----------
Ciona intestinalis	Transparent sea squirt	----------A---------- (SEQ ID NO:4)
Ciona savignyi	Transparent sea squirt	----------AAGUGAUUUAUG (SEQ ID NO:5)

FIG. 32B