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(71) Applicant: ACADEMIA SINICA; 128 Academia Road,
Section 2, Nankang, Taipei, 115 (TW).

(72) Inventors; and

(71) Applicants : SHIH, Chiaho [—/US]; 1815 Orchard
Country Lane, Houston, TX 77062 (US). HUANG, Jyun-
Yuan; No. 6, Ln. 196, Juemin Rd., Sanmin Dist., Ka-
ohsiung City, 807 (TW).

(74) Agent: CHEN, Yahua; Wolf, Greenfield & Sacks, P.C.,
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(54) Title: TREATING DISEASES ASSOCIATED WITH PGC1-ALPHA BY MODULATING MICRORNAS MIR-130A AND
MIR-130B

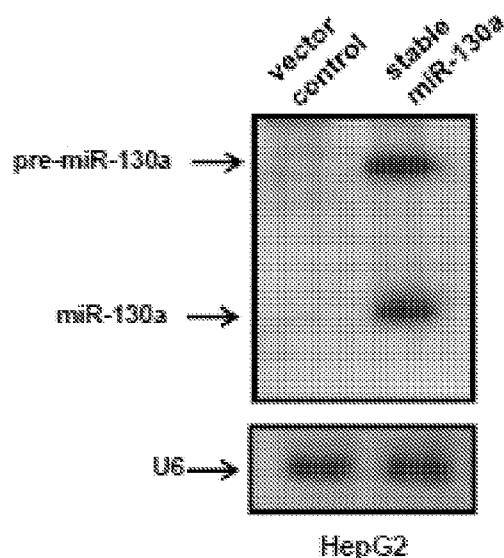


Fig. 1

(57) Abstract: Methods of regulating PGC1 α and/or PGC1 β , or treating diseases associated with PGC1 α and/or PGC1 β using a miR-130a RNA, miR-130b RNA, or a combination thereof, or using an antagomiR of miR-130a, an antagomiR of miR-130b, or both, which can be in the form of microRNA sponge or a locked nucleic acid (LNA).

Treating Diseases Associated with PGC1-Alpha by Modulating MicroRNAs miR-130a and miR-130b

RELATED APPLICATIONS

5 The present application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 61/880,508, filed September 20, 2013, which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

10 Human peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is a protein encoded by the PPARGC1A gene. PGC-1 α is a transcriptional coactivator that regulates genes involved in energy metabolism. It also regulates mitochondrial biogenesis and function. In addition to hepatic gluconeogenesis (Yoon, et al. *Nature* 413:131-138; 2001), PGC1 α is known to be involved in brown adipose adaptive
15 thermogenesis (Puigserver, et al. *Cell* 92:829-839; 1998), mitochondria biogenesis and respiration (Houten, et al. *Cell* 119: 5-7; 2004), and neurodegenerative diseases (St-Pierre, et al. *Cell* 127:397-408; 2006).

MicroRNAs (miRNAs) play an important regulatory role in differentiation and development (Ambros, *Curr Opin Genet Dev* 21:511-517, 2011). Recently, microRNAs
20 have emerged as an important posttranscriptional regulator of metabolism (Rottiers, et al. *Nat Rev Mol Cell Biol* 13:239-250, 2012).

SUMMARY OF THE INVENTION

The present disclosure is based on the unexpected discoveries that miR-130a down
25 regulates PGC1 α , PGC1 β , and PPAR γ by directly targeting the 3'UTR of their mRNAs.

Accordingly, one aspect of the present disclosure features a method for regulating PGC1 α or PGC1 β , the method comprising contacting cells with an effective amount of: (a) a miR-130a RNA, a miR-130b RNA, or a combination thereof; or (b) an antagomiR of the miR-130a RNA, an antagomiR of the miR-130b RNA, or a combination thereof. In some
30 examples, the cells are contacted with the miR-130a, the miR-130b, or a combination thereof in an amount effective to down-regulate PGC1 α or PGC1 β . In other examples, the cells are

contacted with an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof in an amount effective to up-regulate PGC1 α or PGC1 β . An antagomiR of miR-130a, an antagomiR of miR-130b, or both may be microRNA sponges or locked nucleic acid (LNA).

5 In any of the methods described herein, one or more of the miR-130a RNA, the miR-130b RNA are duplex RNA molecules or single-strand RNA molecules. The antagomiR of miR-130a, and antagomiR of miR-130b can be duplex RNA molecules or in the form of a microRNA sponge or LNA. In some embodiments, one or more of the antagomiRs can be a microRNA sponge or an LNA. The miR-130a RNA, the miR-130b RNA, or both may
10 comprise the nucleotide sequence of AGUGCAA. For example, the miR-130a RNA may comprise the nucleotide sequence of CAGUGCAAUGUUAAGGGCAU (SEQ ID NO:1). In another example, the miR-130b RNA may comprise the nucleotide sequence of CAGUGCAAUGAUGAAAGGGCAU (SEQ ID NO:2).

In any of the methods described herein, an effective amount of the miR-130a RNA,
15 the miR-130b RNA, or their antagomiRs thereof may be administered to a subject in need thereof for treating a disease associated with PGC1 α or PGC1 β . Optionally, the disease is not associated with PPAR γ .

In some examples, the subject is a human patient having or suspected of having diabetes or steatosis. The subject may be administered with an effective amount of miR-
20 130a, miR-130b, or both for treating the disease.

In other examples, the subject is a human patient having or suspected of having a disease associated with reactive oxygen species (ROS), such as muscle dysfunction, heart failure, or a neurodegenerative disease (*e.g.*, . Parkinson disease, Huntington disease, or Alzheimer disease). Alternatively, the subject may be a human patient having or suspected of
25 having diabetes or inflammation. Such a patient may be administered with an effective amount of an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof for treating the disease.

Also within the scope of the present disclosure are (a) pharmaceutical compositions for use in treating a disease that is associated with PGC1 α and/or PGC1 β , and may not be

associated with PPAR γ , the composition comprising a pharmaceutically acceptable carrier and one or more of a miR-130a RNA, a miR-130b RNA, or one or more of the antagomiRs of those miRNAs as described herein ; and (b) uses of any of the pharmaceutical compositions or microRNA molecules for manufacturing a medicament for treating any of the target
5 diseases, including, but not limited to, diabetes, steatosis, a disease associated with reactive oxygen species (ROS), such as muscle dysfunction, heart failure, or a neurodegenerative disease (*e.g.*, . Parkinson disease, Hungtinton disease, or Alzheimer disease), and inflammation.

The details of one or more embodiments of the invention are set forth in the
10 description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

15 **Fig. 1.** Expression of miR-130a was detected by Northern blot analysis in HepG2 cell lines stably transfected with miR-130a expression vector.

Fig. 2. Concurrent reduction of PGC1 α and PPAR γ mRNAs in stable miR-130a expressing cell lines was measured by Northern blot analysis.

20 **Fig. 3.** Western blot analysis detected the reduction of PGC1 α , PPAR γ and adiponectin proteins in stable miR-130a expressing cells.

Fig. 4. is a diagram showing the reduction of PGC1 α and PPAR γ mRNA in stable miR-130a expressing HepG2 cell lines as measured by RT-qPCR analysis (upper panel). No appreciable difference in the protein levels of SP1, PPAR α , CEBP β , and HNF4 by Western blot analysis (lower panel).

25 **Fig.5.** The reduction of secreted adiponectin was also detected by ELISA.

Fig. 6. HepG2 cells were transfected with LNA-miR-130a or a LNA scramble control. Treatment by LNA-miR-130a increased PGC1 α mRNA and protein by real time RT-qPCR (*upper panel*) and Western blot analysis (*lower panel*), respectively.

Fig. 7. Transgenic mice exhibited increased expression of PGC1 α , PEPCK, G6Pase, PPAR γ and adiponectin protein in the liver (*left panel*) and adiponectin in sera (*right panel*).

Fig. 8. Cotransfection of both PGC1 α and PPAR γ expression vectors resulted in a highly potent synergistic effect on adiponectin secretion.

Fig. 9. is a diagram showing that MiR-130a significantly reduced the luciferase activity in a reporter cotransfection assay of the 3' UTR of PGC1 α (NM_013261) and PPAR γ (NM_005037), but not the 3' UTR of SP1 (NM_003109).

Fig. 10. MiR-130a was shown to directly target at the 3' UTR of PGC1 α by compensatory mutagenesis. (*p < 0.05). Mutation sites were underlined in sequence alignment. hsa-miR-130a WT: SEQ ID NO:1; hsa-PGC1 α 3'UTR WT: SEQ ID NO: 68; hsa-miR-130a seed sequence *mutant*: SEQ ID NO: 69; and hsa-PGC1 α 3'UTR target site mutant: SEQ ID NO:70.

Fig. 11. is a diagram showing the co-transfection of the PGC1 α 3'UTR luciferase reporter with increasing amounts of miR-130a resulted in decreasing reporter activity in a dose response manner (*upper panel*). Conversely, treatment with increasing amounts of LNA-miR-130a plasmid resulted in increasing reporter activity in a dose response manner (*lower panel*).

Fig. 12. This diagram highlights the key enzymes in glucose metabolism. The mRNA expression of G6Pase, and PEPCK was reduced in stable miR-130a expressing HepG2 cells by Northern blot analysis.

Fig. 13. Reduced protein expression of PEPCK and G6Pase was also detected by Western blot analysis. We noted an increased level of glucokinase (GCK) in stable miR-130a expressing HepG2 cells, and an increased level of pyruvate kinase (PKLR) in stable miR-130a expressing Huh7 cells.

Fig. 14. The levels of PKLR and GCK specific mRNAs were increased in stable miR-130a expressing cells by RT-qPCR analysis.

Fig. 15. The protein expression of PGC1 α and gluconeogenic enzymes PEPCK and G6Pase in HBV-producing HepG2 cells (UP7-4 and UP7-7) was increased by Western blot analysis. However, no significant change in PPAR γ was noted.

Fig.16. Stable expression of PPAR γ in HepG2 cells reduced glucose output, and conversely, expression of PGC1 α stimulated glucose production.

Fig. 17. Both stable HBV replication and a PPAR γ antagonist, GW9662 (20 μ M), can increase glucose production in HepG2 cells.

5 **Fig. 18.** The expression of miR-130a was not affected in two stable PGC1 α - expressing cell lines.

Fig. 19. This diagram summarizes the relationships among PGC1 α , miR-130a and HBV. +/-: neither positive nor negative effect.

Fig 20. *upper panel:* Reduction of miR-130a was observed in PPAR γ -expressing
10 HepG2 cell lines using stem-loop qPCR. U6 snRNA was used as an internal control. Rosiglitazone, but not GW9662, further reduced the expression of miR-130a. *lower panel:* Increased amounts of PPAR γ protein in stable PPAR γ -expressing HepG2 cell lines were detected by Western blot.

Fig. 21. This triad cartoon summarizes the relationships among PPAR γ , PGC1 α , miR-
15 130a and HBV. A feed-forward amplification loop among HBV and PGC1 α and PPAR γ can be mediated through a miR-130a intermediate.

Fig. 22 is a schematic illustration showing therapeutic strategies.

DETAILED DESCRIPTION OF THE INVENTION

20 The present disclosure is based on the unexpected discoveries that miR-130a regulates PGC1 α , PGC1 β , and PPAR γ by direct targeting the 3' UTR of their messenger RNAs. As such, miR-130 microRNAs (*e. g.*, miR-130a and miR-130b), as well as their antagomiRs can be used to modulate these proteins and thus be effective in treating diseases associated with one or more of these proteins (*e.g.*, diseases associated with ROS or diseases associated with
25 dysfunction of mitochondria). Also, the simultaneous effect of miR-130a on PGC1 α (gluconeogenesis) and PPAR γ (inhibition of gluconeogenesis) may contribute to glucose homeostasis.

A miR-130a RNA, a miR-130b RNA, or an antagomiR thereof may be used to modulate the activity of the corresponding microRNA and thus its target gene expression.

Modulating a microRNA means any approach that affects the ultimate biological function of the microRNA in regulating its target gene expression. In some examples, modulating a microRNA is to regulate the cellular level of the microRNA. In other examples, modulating a microRNA is to regulate (*e.g.*, enhance or block) its interaction with a target of the microRNA (*e.g.*, a mRNA or a gene).

Accordingly, described herein are methods for relating PGC1 α , PGC1 β , or both, or treating a disease associated with one or more of the proteins using a miR-130a RNA, a miR-130b RNA, or both, or an antagomiR of miR-130a or miR-130b, as well as pharmaceutical compositions for use in the treatments described herein and for use in manufacturing medicaments for those purposes. miR-130b contains the same AGUGCAA sequence as miR-130a for base pairing with a target gene.

MicroRNA molecules

MicroRNAs are small non-coding RNA molecules (*e.g.*, 22 nucleotides) found in many species, which regulates gene expression. miR-130a and miR-130b are found in many species, *e.g.*, human. The nucleotide sequences of exemplary miR-130a and miR-130b (precursor and mature) can be found in MiRBase under accession numbers MI0000448 (human miR-130a) and MI0000748 (human miR-130b). Exemplary nucleotide sequences of these miRNA molecules are provided below:

Human miR-130a:

ugcugcuggc cagagcucuu uucacauugu gcuacugucu gcaccuguca
cuagcagugc aauguuaaaa gggcauuggc cguguagug (SEQ ID NO:3)

Human miR-130b:

ggccugcccg acacucuuuc ccuguugcac uacuauaggc cgcugggaag
cagugcaaug augaaagggc aucggucagg uc (SEQ ID NO:4)

A miR-130a RNA as described herein is an oligonucleotide (*e.g.*, an RNA molecule) that possesses the same bioactivity as a wild-type miR-130a, such as the human miR-130a, *e.g.*, regulating the expression of PGC1 α , PGC1 β , and/or PPAR γ . Such an oligonucleotide

can comprise the nucleotide sequence of miR-130a or a portion thereof (*e.g.*, AGUGCAA or CAGUGCAAUGUUAAAAGGGCAU (SEQ ID NO:1)). A miR-130a RNA can include up to 150 (*e.g.*, 100, 80, 60, 50, 40, 30, or less) nucleotide residues. In some examples, the miR-130a can be a duplex RNA molecule. In other examples, it can be a hairpin molecule, which may include a 21-23 sense sequence (*e.g.*, CAGUGCAAUGUUAAAAGGGCAU (SEQ ID NO:1)), a short linker, an antisense sequence complementary to the sense sequence, and a polyT tail.

A miR-130b RNA as described herein is an oligonucleotide such as an RNA molecule that possesses the same bioactivity as a wild-type miR-130b, such as the human miR-130b. Since miR-130b share the same sequence as miR-130a for base pairing with target genes, miR-130b would possess the same biological functions as miR-130a, *e.g.*, regulating the expression of PGC1 α , PGC1 β , and/or PPAR γ . Such an oligonucleotide can comprise the nucleotide sequence of miR-130b or a portion thereof (*e.g.*, AGUGCAA or CAGUGCAAUGAUGAAAGGGCAU (SEQ ID NO:2)). A miR-130b RNA can include up to 150 (*e.g.*, 100, 80, 60, 50, 40, 30, or less) nucleotide residues. In some examples, the miR-130b can be a duplex RNA molecule. In other examples, it can be a hairpin molecule, which may include a 21-23 sense sequence (*e.g.*, CAGUGCAAUGAUGAAAGGGCAU (SEQ ID NO:2)), a short linker, an antisense sequence complementary to the sense sequence, and a polyT tail.

An antagomiR of miR-130a or miR-130b can be an engineered oligonucleotide capable of suppressing the activity of the target (*e.g.*, endogenous) miR-130a or miR-130b in a cell via, *e.g.*, blocking the binding of the target miRNA to a target mRNA molecule. An antagomiR can be a small synthetic oligonucleotide that is completely or partially complementary to the target miRNA or a portion thereof with either mis-pairing at, *e.g.*, the cleavage site of Ago2. It may also include some modifications to inhibit Ago cleavage. In some examples, an antagomiR can include up to 150 (*e.g.*, 100, 80, 60, 50, 40, 30, or less) nucleotide residues. In some examples, an antagomiR can be a microRNA sponge, which can be transcripts expressed from strong promoters and containing multiple, tandem binding sites to a microRNA of interest. When vectors encoding these sponges are transiently transfected into cultured cells, sponges would suppress microRNA targets at least as strongly as

chemically modified antisense oligonucleotides. They specifically inhibit microRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire microRNA seed family. RNA polymerase II promoter (Pol II)-driven sponges contain a fluorescence reporter gene for identification and sorting of sponge-treated cells. Stably
5 expression of such microRNA sponges may be used in disease treatment. Ebert et al., *Nat Methods*. 2007 Sep;4(9):721-6. Epub 2007 Aug 12).

When necessary, the microRNA molecules or their antagomiRs can include non-naturally-occurring nucleobases, sugars, or covalent internucleoside linkages (backbones). Such a modified oligonucleotide confers desirable properties such as enhanced cellular
10 uptake, improved affinity to the target nucleic acid, and increased *in vivo* stability.

In one example, the oligonucleotide/RNA molecules described herein has a modified backbone, including those that retain a phosphorus atom (see, e.g., U.S. Pat. Nos. 3,687,808; 4,469,863; 5,321,131; 5,399,676; and 5,625,050) and those that do not have a phosphorus atom (see, e.g., U.S. Pat. Nos. 5,034,506; 5,166,315; and 5,792,608). Examples of
15 phosphorus-containing modified backbones include, but are not limited to, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates,
20 thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having 3'-5' linkages, or 2'-5' linkages. Such backbones also include those having inverted polarity, i.e., 3' to 3', 5' to 5' or 2' to 2' linkage. Modified backbones that do not include a phosphorus atom are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl
25 internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. Such backbones include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate
30 backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide

backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

In another example, the microRNA molecules or antagomiR molecules described herein includes one or more substituted sugar moieties. Such substituted sugar moieties can include one of the following groups at their 2' position: OH; F; O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl; O-alkynyl, S-alkynyl, N-alkynyl, and O-alkyl-O-alkyl. In these groups, the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. They may also include at their 2' position heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide. Preferred substituted sugar moieties include those having 2'-methoxyethoxy, 2'-dimethylaminoethoxy, and 2'-dimethylaminoethoxyethoxy. See Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504.

In yet another example, the microRNA molecules or antagomiRs described herein includes one or more modified native nucleobases (*i.e.*, adenine, guanine, thymine, cytosine and uracil). Modified nucleobases include those described in U.S. Pat. No. 3,687,808, The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613, and Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-302, CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the microRNA molecules to their targeting sites. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines (e.g., 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine). See Sanghvi, et al., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278).

In some examples, any of the antagomiR molecules are locked nucleic acids (LNA). LNA is a modified RNA molecule containing at least one modified RNA nucleotide, in which the ribose moiety is modified with an extra bridge connecting the 2' oxygen and 4' carbon so as to lock the ribose in the 3' endo confirmation. See, *e.g.*, Janssen et al., *N Engl J Med.* 368(18):1685-94 (2013), Lanford et al., *Science* 327(5962):198-201 (2010); . Hildebrandt-Eriksen et al., *Nucleic Acid Ther.* 22(3):152-61 (2013); and . Ørom et al., *Gene*.

372:137-41 (2006). In some embodiments, an LNA as described herein is a modified DNA phosphorothioate antisense oligonucleotide to either miR-130a or miR-130b.

Any of the microRNAs and antagomiRs described herein can be prepared by conventional methods, *e.g.*, chemical synthesis or *in vitro* transcription. Their intended bioactivity as described herein can be verified by routine methods, *e.g.*, those described in the Examples below.

Use of miR-130 or AntagomiR thereof in Modulating PGC1 α or PGC1 β

The miR-130a RNA, the miR-130b RNA, or an antagomiR of miR-130a or miR-130b as described herein, either alone or in combination, can be used in modulating PGC1 α and/or PGC1 β and in treating diseases associated with one or both of the proteins. For example, miR-130a and/or miR-130b can be used to down-regulate PGC1 α and/or PGC1 β , thereby be effective in treating diseases where down-regulation of PGC1 α and/or PGC1 β is needed. Alternatively, an antagomiR of miR-130a, an antagomiR of miR-130b, or both can be used to up-regulate PGC1 α and/or PGC1 β , thereby be effective in treating diseases where up-regulation of PGC1 α and/or PGC1 β is needed. PGC1 α can function as a detoxifying enzyme for reactive oxygen species (ROS), (St Pierre et al., (2006) Cell 127:page 397-408). ROS is responsible for a large number of human diseases, such as cancer, neurodegenerative disease (Parkinson or Huntington disease), heart failure, muscle dysfunction. Thus, an antagomiR of miR-130a or miR-130b can be used for treating such diseases. Examples of such diseases and their association with PGC1 α are illustrated in Fig.23.

The term “treating” as used herein refers to the application or administration of a composition including one or more active agents to a subject, who has a disease associated with PGC1 α , PGC1 β , or both, a symptom of the disease, or a predisposition toward the disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease.

To perform the method described herein, an effective amount of the miR-130a RNA, the miR-130b RNA, or both, or an effective amount of an antagomiR of miR-130a, an antagomiR of miR-130b, or both, can be in contact with cells, in which regulation of PGC1 α and/or PGC1 β is needed. In some embodiments, the method can be performed *in vitro*, *e.g.*,

in cultured cells.

In other embodiments, an effective amount of one or more microRNA molecules or antagomiRs as described herein can be administered to a subject in need of the treatment via a suitable route. Such a subject can be a human patient having, suspected of having, or at risk for a disease associated with dysregulation of PGC1 α , PGC1 β , or both. In some
5 embodiments, the disease is not associated with PPAR γ . The one or more microRNA molecules or antagomiRs can be administered to a subject in need of the treatment directly or indirectly (e.g., using naked oligonucleotides or using expression vectors adapted for expressing the microRNA molecules or antagomiRs). Such an expression vector can be
10 constructed by inserting one or more nucleotide sequences of the microRNA(s) or one or more antagomiRs into a suitable expression vector, in which the microRNA sequences are in operable linkage with a suitable promoter.

One or more of the miR-130a RNA and miR-130b RNA, or one or more of the antagomiRs of miR-130a and miR-130b, or one or more expression vectors suitable for
15 expressing such can be mixed with a pharmaceutically acceptable carrier to form a pharmaceutical composition. An "acceptable carrier" is a carrier compatible with the active ingredient of the composition (and preferably, stabilizes the active ingredient) and not deleterious to the subject to be treated. Suitable carriers include, but are not limited to, (a) salts formed with cations (e.g., sodium, potassium, ammonium, magnesium, calcium) and
20 polyamines (e.g., spermine and spermidine); (b) acid addition salts formed with inorganic acids (e.g., hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid); (c) salts formed with organic acids (e.g., acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid,
25 methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid); and (d) salts formed from elemental anions (e.g., chlorine, bromine, and iodine). Other suitable carriers include microcrystalline cellulose, mannitol, glucose, defatted milk powder, polyvinylpyrrolidone, starch, and a combination thereof. See, e.g., Remington's Pharmaceutical Sciences, Edition 18, Mack Publishing Co., Easton, Pa (1995); and Goodman

and Gilman's "The Pharmacological Basis of Therapeutics," Tenth Edition, Gilman, J. Hardman and L. Limbird, eds., McGraw-Hill Press, 155-173, 2001.

To facilitate delivery, the microRNA molecules, the antagomiRs, or the expression vectors thereof can be conjugated with a chaperon agent. As used herein, "conjugated" means two entities are associated, preferably with sufficient affinity that the therapeutic benefit of the association between the two entities is realized. Conjugated includes covalent or noncovalent bonding as well as other forms of association, such as entrapment of one entity on or within the other, or of either or both entities on or within a third entity (*e.g.*, a micelle).

The chaperon agent can be a naturally occurring substance, such as a protein (*e.g.*, human serum albumin, low-density lipoprotein, or globulin), carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid), or lipid. It can also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolid) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl) methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, and polyphosphazine.

In one example, the chaperon agent is a micelle, liposome, nanoparticle, or microsphere, in which the microRNA molecules or expression vectors are encapsulated. Methods for preparing such a micelle, liposome, nanoparticle, or microsphere are well known in the art. See, *e.g.*, U.S. Pat. Nos. 5,108,921; 5,354,844; 5,416,016; and 5,527,5285.

In another example, the chaperon agent serves as a substrate for attachment of one or more of a fusogenic or condensing agent.

A fusogenic agent is responsive to the local pH. For instance, upon encountering the pH within an endosome, it can cause a physical change in its immediate environment, *e.g.*, a change in osmotic properties which disrupts or increases the permeability of the endosome membrane, thereby facilitating release of the microRNA described herein into host cell's cytoplasm. A preferred fusogenic agent changes charge, *e.g.*, becomes protonated at a pH

lower than a physiological range (*e.g.*, at pH 4.5-6.5). Fusogenic agents can be molecules containing an amino group capable of undergoing a change of charge (*e.g.*, protonation) when exposed to a specific pH range. Such fusogenic agents include polymers having polyamino chains (*e.g.*, polyethyleneimine) and membrane disruptive agents (*e.g.*, mellittin). Other
5 examples include polyhistidine, polyimidazole, polypyridine, polypropyleneimine, and a polyacetal substance (*e.g.*, a cationic polyacetal).

A condensing agent interacts with any of the microRNAs, any of the antagomiRs, or any of the expression vectors for expressing such, causing it to condense (*e.g.*, reduce the size of the oligonucleotide), thus protecting it against degradation. Preferably, the condensing
10 agent includes a moiety (*e.g.*, a charged moiety) that interacts with the oligonucleotide via, *e.g.*, ionic interactions. Examples of condensing agents include polylysine, spermine, spermidine, polyamine or quarternary salt thereof, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, and alpha helical peptide.

15 In some embodiments, an effective amount of a miR-130a RNA, a miR-130b RNA, or a combination thereof is administered to a subject (*e.g.*, a human patient) suffering from, suspected of having, or at risk for a disease associated with abnormally up-regulated PGC1 α , PGC1 β , or both. Such diseases include, but are not limited to, diabetes and steatosis. See Fig. 22. In some examples, the amount of the microRNA or an expression vector for
20 producing such is effective to down-regulate PGC1 α , PGC1 β , or both.

In other embodiments, an effective amount of an antagomiR of miR-130a or miR-130b, or an expression vector for producing such is administered to a subject (*e.g.*, a human patient) suffering from, suspected of having, or at risk for a disease associated with abnormally down-regulated PGC1 α , PGC1 β , or both. Such diseases include, but are not
25 limited to a disease associated with ROS (*e.g.*, a neurodegenerative disease such as Parkinson disease, Huntington disease, or Alzheimer disease, inflammation, muscle dysfunction, a cardiovascular disease such as heart failure. See Fig. 22. In some examples, the amount of the microRNA or an expression vector for producing such is effective to up-regulate PGC1 α , PGC1 β , or both.

“An effective amount” as used herein refers to the amount of a microRNA molecule, an antagomiR thereof, or an expression vector for expressing such, that alone, or together with further doses or one or more other active agents, produces the desired response, *e.g.*, enhancing or inhibiting PGC1 α , PGC1 β , or both, and/or alleviating one or more symptoms of a target disease (*e.g.*, those described herein). This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods, such as physical examination and suitable lab tests. The desired response to treatment of any of the target diseases disclosed herein also can be delaying the onset or even preventing the onset of the disease.

Effective amounts vary, as recognized by those skilled in the art, depending on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size, gender and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The interrelationship of dosages between animals and humans (*e.g.*, based on milligrams per meter squared of body surface or milligrams per body weight) is well known in the art. See, *e.g.*, Freireich *et al.*, (1966) *Cancer Chemother Rep* 50: 219. Body surface area may be approximately determined from height and weight of the patient.

A subject in need of any of the above-described treatments can be a subject (*e.g.*, a human) suffering from, suspected of having, or at risk for developing any of the target diseases described herein. Examples include, but are not limited to, diabetes (such as type I diabetes, type II diabetes, as well as syndromes associated with diabetes such as retinopathy), a disease associated with ROS such as muscle dysfunction, heart failure, and

neurodegenerative diseases (*e.g.*, Parkinson's disease, Huntington's disease). Such a subject can be identified via a routine medical procedure, including, but are not limited to, physical examination and pathological analysis. Common symptoms of diabetes include, but are not limited to, frequent urination, feeling thirsty, feeling hungry, unusual weight loss or weight gain, fatigue, and blurred vision. Such subjects can be identified via routine medical procedures. A subject at risk for developing a disease as described herein, such as a disease associated with ROS possesses one or more risk factors associated with the disease or disorder.

Conventional methods, known to those of ordinary skill in the art of medicine, can be used to administer to a subject in need of the treatment the pharmaceutical composition described above. For example, the pharmaceutical composition described above can be delivered orally or parenterally. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial administration (*e.g.*, intrathecal or intraventricular).

An injectable composition containing an microRNA molecule described herein, an antagomiR described herein, or an expression vector thereof may contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, and polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injection, the oligonucleotide can be administered by the drip method, whereby a pharmaceutical formulation containing the oligonucleotide and a physiologically acceptable excipients is infused. Physiologically acceptable excipients may include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, *e.g.*, a sterile formulation of a suitable soluble salt form of a peptide, can be dissolved and administered in a pharmaceutical excipient such as sterile water, 0.9% saline, or 5% glucose solution.

When oral administration is applied, it is preferred that the oligonucleotide includes at least one 2'-O-methoxyethyl modification. A composition for oral administration can be any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as

magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavoring, or coloring agents can be added. A nasal aerosol or inhalation composition can be prepared according to techniques well known in the art of pharmaceutical formulation. The pharmaceutical composition described herein can also be administered in the form of suppositories for rectal administration.

Kits

The present disclosure also provides kits for use in regulating (*e.g.*, inhibiting or enhancing) PGC1 α , PGC1 β , and/or PPAR γ , or treating a disease associated with PGC1 α , PGC1 β , or both as those described herein. Such kits can include one or more containers comprising one or more of the microRNA molecules, the antagomiRs, or expression vectors thereof.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the microRNA(s), the antagomiRs, or the expression vector(s) thereof to treat a desired target disease. The kit may further comprise a description of selecting an individual suitable for treatment based on identifying whether that individual has the target disease.

The instructions relating to the use of an microRNA or an antagomiR as described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine-readable instructions (*e.g.*, instructions carried on a magnetic or optical storage disk) are also acceptable.

The label or package insert indicates that the composition is used for inhibiting or enhancing PGC1 α , PGC1 β , or both, and for treating any of the target diseases described

herein may be provided for practicing any of the methods described herein.

The kits of this invention are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device (*e.g.*, an atomizer) or an infusion device such as a minipump. A kit may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a microRNA molecule or its expression vector as described herein.

Kits may optionally provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container. In some embodiments, the invention provides articles of manufacture comprising contents of the kits described above.

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

Examples: Regulation of PGC1-alpha With MiR-130

MATERIALS AND METHODS

Construction of miRNA Plasmids

The sequences of human miRNAs were retrieved from Ensembl database and miRbase (Version 16) as noted above. The primer sequences used in cloning the full length precursor miRNAs are listed in Table 1 below:

Table 1. DNA sequences of synthetic oligonucleotides and PCR primers used in this study

Primer name	sequences	SEQ ID NO:
hsa-miR-31-F	5'-CATCTTCAAAAGCGGACACTC-3'	5
hsa-miR-31-R	5'-TCATGGAAATCCACATCCAA-3'	6
hsa-miR-130a-F	5'-GGCAAAAGGAAGAGTGGTGA-3'	7
hsa-miR-130a-R	5'-ACCAGGGTAGCTGACTGGTG-3'	8
HBV ayw nt 1521-2122 F	5'-AGCAGGTCTGGAGCAAACAT-3'	9
HBV ayw nt 1521-2122 R	5'-CACCCACCCAGGTAGCTAGA-3'	10
HBV ayw nt 1521-2122 mt F	5'-GGAGGAGTTGGGAGAGGAAATTAGGTTAAAGG-3'	11
HBV ayw nt 1521-2122 mt R	5'- CCTTTAACCTAATTTCTCTCCCAACTCCTCC-3'	12
hsa-PGC1a 3'UTR-F	5'- ATATTCTAGAGCTTGTTTCAGCGGTTCTTTC-3'	13
hsa-PGC1a 3'UTR-R	5'- ATAT TCTAGA AGCCATCAAGAAAGGACACA-3'	14
hsa-PGC1a 3'UTR mt-F	5'- GCAGTGTTCCTACTTGCTCAAGCATGGCCTCT-3'	15
hsa-PGC1a 3'UTR mt-R	5'- AGAGGCCATGCTTGAGCAAGTAGAAACACTGC-3'	16
hsa-miR-130a mt-F	5'- GCACCTGTCACTAGCTGAGCAATGTTAAAAGG-3'	17
hsa-miR-130a mt-R	5'- CCTTTTAACATTGCTCAGCTAGTGACAGGTGC-3'	18
miR-130a sponge-F	5'- GCACTGCTCGAGATGCCCTTTTAACATTGCACTGGAAT TCATGCCCTTTTAACATTGCACTGCTCGAGATGCC-3'	19
miR-130a sponge-R	5'- GCATCTCGAGCAGTGCAATGTTAAAAGGGCATGAATT CCAGTGCAATGTTAAAAGGGCATCTCGAGCAGTGTC-3'	20
hsa-SP1 3'UTR-F	5'- ATATCTCGAGAGATGCATTCACAGGGGTTg-3'	21
hsa-SP1 3'UTR-R	5'-ATATCTCGAGGCTCAGAGCAGCTAATGAAG-3'	22
hsa-PPARg 3'UTR-F	5'-ATATCTCGAGCAGAGAGTCCTGAGCCACT -3'	23
hsa-PPARg 3'UTR-R	5'-ATATCTCGAGGGGTGGGAAACACACAAGA-3'	24
Q-PCR hsa-SOD2-F	5'-CCACTGCTGGGGATTGATGT-3'	25
Q-PCR hsa-SOD2-R	5'-GAGCTTAACATACTCAGCATAACG-3'	26
Q-PCR hsa-GPx1-F	5'-GCGGGGCAAGGTACTACTTAT-3'	27
Q-PCR hsa-GPx1-R	5'-CGTTCTTGCGTTCTCCTGA-3'	28
Q-PCR hsa-CYCS-F	5'-GGAGCGAGTTTGGTTGCACT-3'	29
Q-PCR hsa-CYCS-R	5'-GTGGCACTGGGAACACTTCA-3'	30
Q-PCR hsa-Acly-F	5'-CCTGCCATGCCACAAGATTC-3'	31
Q-PCR hsa-Acly-R	5'-TCTGCATGCCCCACACAAT-3'	32
Q-PCR hsa-ApoE-F	5'-CCTTCCCCAGGAGCCGAC-3'	33
Q-PCR hsa-ApoE-R	5'-GCTCTGTCTCCACCGCTT-3'	34
Q-PCR hsa-GCK-F	5'-TACATGGAGGAGATGCAGAATg-3'	35
Q-PCR hsa-GCK-R	5'-ACTTGCCACCTATGAGCTTCTC-3'	36
Q-PCR hsa-PKLR-F	5'-GAGATCCCAGCAGAGAAGGTTT-3'	37
Q-PCR hsa-PKLR-R	5'-AGTCTCCCCTGACAGCATGA-3'	38
Q-PCR hsa-PPARg-F	5'-CATAAAGTCCTTCCCGCTGA-3'	39

Q-PCR hsa-PPAR γ -R	5'-TCTGTGATCTCCTGCACAGC-3'	40
Q-PCR hsa- HNF1- F	5'-ACCTCATCATGGCCTCACTT-3'	41
Q-PCR hsa-HNF1 - R	5'-GTTGATGACCGGCACACTC-3'	42
Q-PCR hsa- HNF4-F	5'- GAGCTGCAGATCGATGACAA-3'	43
Q-PCR hsa-HNF4-R	5'-TACTGGCGGTCTGTTGATGTA-3'	44
Q-PCR hsa-PPAR α -F	5'-CCTCTCAGGAAAGGCCAGTA-3'	45
Q-PCR hsa-PPAR α -R	5'-CACTTGATCGTTCAGGTCCA -3'	46
Q-PCR hsa- ESRR γ -F	5'-GGAGAACAGCCCATACCTGA -3'	47
Q-PCR hsa- ESRR γ - R	5'- GCCCATCCAATGATAACCAC-3'	48
Q-PCR hsa- CEBP β -F	5'-GACAAGCACAGCGACGAGTA -3'	49
Q-PCR hsa- CEBP β - R	5'-AGCTGCTCCACCTTCTTCTG -3'	50
Q-PCR hsa- CEBP α -F	5'- CAGACCACCATGCACCTG-3'	51
Q-PCR hsa- CEBP α -R	5'-CTCGTTGCTGTTCTTGTCCA -3'	52
Q-PCR hsa- SP1-F	5'-GCACCTGCCCCTACTGTAAA -3'	53
Q-PCR hsa- SP1-R	5'- GCGTTTCCCACAGTATGACC-3'	54
Q-PCR hsa- ESRR α -F	5'- TGGCTACCCTCTGTGACCTC-3'	55
Q-PCR hsa- ESRR α -R	5'-CCCCTCTTCATCCAGGACTA -3'	56
Q-PCR hsa- LRH-F	5'- ATCCTCGACCACATTTACCG-3'	57
Q-PCR hsa- LRH-R	5'- TGCCACTAACTCCTGTGCAT -3'	58
Q-PCR hsa-PGC1 α -F	5'- TATCAGCACGAGAGGCTGAA-3'	59
Q-PCR hsa-PGC1 α -R	5'- TCAAAACGGTCCCTCAGTTC-3'	60
Q-PCR hsa-Scd1-F	5'- GCAAACACCCAGCTGTCAAA-3'	61
Q-PCR hsa-Scd1-R	5'- GCACATCATCAGCAAGCCAG -3'	62
Q-PCR hsa-Acsl1-F	5'-GAGTGGGCTGCAGTGACA -3'	63
Q-PCR hsa-Acsl1-R	5'-GCACGTACTGTCTGGAAGTCA -3'	64

The methods to construct the miRNA expression vectors are as detailed elsewhere. Chen, H.L. et al. *PloS one* **7**, e34116 (2012). PCR products were sub-cloned from TA cloning vector (RBC) to pSuper (OligoEngine, Inc) by Hind III digestion. All plasmids were confirmed by sequencing. Approximately 8-400 fold higher level of microRNA expression was detected by transfection and stem loop RT-PCR analysis. The PPAR γ and PGC1 α expression vectors were from GeneCopoeia.

Source of antibodies

Anti-HBc (Dako), anti-PPAR γ (Santa Cruz), anti-GAPDH, anti-PKLR, anti-G6Pase, anti-tubulin, anti-adiponectin (GeneTex, Taiwan), anti-PGC1 α (Origene), anti-PCK1 (Abnova), anti-GCK (Biovision), Secondary antibodies include mouse anti-rabbit-HRP, goat anti-mouse-HRP (GeneTex, Taiwan) and donkey anti-goat-HRP (Santa Cruz).

Synthetic RNA

The synthetic miRNAs (Genepharma) used are RNA duplexes without modifications. The sense strand is the same as the mature form of miRNAs. Mimic miR-130a (CAGUGCAAUGUAAAAGGGCAU (SEQ ID NO:1)), mimic miR-204 (UUCCCUUUGUCAUCCUAUGCCU (SEQ ID NO:65)), mimic miR-1236 (CCUCUUCCCCU UGUCUCUCCAG (SEQ ID NO:66)), mimic negative control (UUCUCCGAACGUGUCACGUTT (SEQ ID NO:67)). The siRNA oligo against PGC1 α was from Dharmacon.

MiR-130a sponge

Each sense and antisense oligos (see Table 1 above) were designed to contain four copies of synthetic target sites of miR-130a. Annealed oligo product was gel purified before PCR amplification. Gel-purified PCR product was subcloned into DsRedC1 vector at Hind III site. Colonies were screened by PCR and the orientation of the insert and the copy numbers of target sites were confirmed by sequencing.

Cell Culture

Human hepatoma Huh7 and HepG2 cells were maintained as described previously Chua, P.K., et al. *Journal of virology* 84, 2340-2351 (2010), Le Pogam, S., et al., *Journal of virology* 79, 1871-1887 (2005). In general, the phenotype of viral replication and the effect of microRNA are stronger in HepG2 than Huh7 cells. However, Huh7 cells are easier to passage and transfect. Therefore, we used these two cell lines interchangeably.

PPAR γ agonist and antagonist

Rosiglitazone and GW9662 were from Sigma. HepG2 and Huh7 cells were seeded in 6-well tissue culture plates at 5x10⁵, Quasdorff, M. et al. *Journal of viral hepatitis* 17, 527-536 (2010) cells/well. At 24 h post-transfection, Rosiglitazone or GW9662 in 0.1% DMSO was added to medium. Culture medium was changed ever two days before harvest.

ELISA

The concentration of secreted adiponectin was measured by ELISA (Biovision).

ELISA of HBsAg and HBeAg was from General Biologicals Co., Taiwan.

Measurement of glucose production

The glucose level of HBV transgenic mice was measured using a kit of DRI-CHEM SLIDE GLU-PIII (FUJIFILM, JAPAN). Ten microliters of mouse serum was deposited on a FUJI DRI-CHEM SLIDE GLU-PIII. Glucose oxidase (GOD) catalyzes the oxidization of sample glucose to generate hydrogen peroxide which then reacts with dye precursors and forms red dye. The optical reflection density was measured at 505 nm by the FUJI DRI-CHEM analyzer and converted into the glucose concentration (mg/ L). For the measurement of glucose concentration in cell culture, HBV producing cells (HepG2 and Q7 cells) were treated with PPAR γ antagonist, GW9662 at indicated concentrations. Twenty-four hours before glucose measurement, the medium was replaced with 1ml of glucose-free DMEM, supplemented with 2mM sodium pyruvate. After 16hrs incubation, 50 μ l of medium was collected and the glucose concentration (mM) was measured using the glucose colorimetric assay (Biovision).

Quantitative Real-time PCR

Briefly, 2 μ g of total RNA was reverse transcribed into cDNA using random primers and High Capacity cDNA Reverse Transcription kit (Applied Biosystem) at 37 °C for 120 minutes. The cDNA product was then diluted 100 times for real-time PCR analysis using Power SYBR Green PCR master mix (Applied Biosystem), and the default condition in a 20 μ l reaction volume by Applied Biosystems 7500 Real-Time PCR System. Data were analyzed by relative quantification methods ($\Delta\Delta$ Ct methods) using 7500 software V2.0.1.

Stem-loop qCR for miRNA

Taqman RT and stem-loop real-time assay were from Applied Biosystems: miR-31 (assayID: 002279), miR-130a (assayID: 000454), miR-204 (assayID: 000508) and miR-1236 (assayID: 002752). Briefly, 100 ng RNAs were reverse transcribed by specific stem-loop primer and further analyzed by Taqman real-time PCR assay using default setting. U6 snRNA (assayID: 001973) was used as an internal loading control. Data were analyzed by Applied Biosystems 7500 software V2.0.1.

Southern and Northern blot

HBV core particle-associated DNA, total cellular cytoplasmic RNA, and microRNA were analyzed by Northern blot as described previously. Chua, P.K., et al. *Journal of virology* 84, 2340-2351 (2010), Le Pogam, S., et al., *Journal of virology* 79, 1871-1887 (2005).

5 *Luciferase reporter assay*

Assay for 3' UTR or enhancer/promoter was as described previously. Chen, H.L., et al., *PloS one* 7, e34116 (2012).

Native agarose gel electrophoresis and Western blot

Native agarose gel electrophoresis and Western blot for detecting HBV core particles were as described. Chua, P.K., et al. *Journal of virology* 84, 2340-2351 (2010),

Stable miR-130a expressing cell lines

Approximately one million Huh7 and HepG2 cells were transiently transfected by 3µg plasmid DNA (pSuper and pSuper-miR-130a) with Polyjet (SignaGen), followed by G418 selection for three weeks. The G418-resistant colonies were pooled together.

15 *LNA-miR-130a knockdown*

HepG2 and Huh7 cells were cotransfected with puromycin resistant plasmid (pTRE2pur) and LNA-scramble control or LNA anti-miR-130a (Locked Nucleic Acid, Exiqon), using Lipofectamine 2000 (Invitrogen). Twelve hours post-transfection, transfected culture was treated with puromycin (2 µg/ml) for 2 days, followed by reduced concentration of puromycin (0.5 µg/ml) for another 2 days before harvesting for Western blot analysis. Chen, H.L., et al., *PloS one* 7, e34116 (2012).

Bioinformatic analysis.

Computer-based programs including Targetscan (<http://www.targetscan.org/>), Pictar (<http://pictar.mdc-berlin.de/>), Microinspector (<http://bioinfo.uni-plovdiv.bg/microinspector/>), RNAhybrid (<http://www.bibiserv.techfak.uni-bielefeld.de/>) and DIANA (<http://diana.cslab.ece.ntua.gr>) were used to predict potential targets for miR-1236, miR-130a and miR-204. The minimal free energy of binding less than -20 kcal/ mol was used as the cut-off value.

MicroRNA Taqman low density array analysis

The total RNA of HBV-producing cells were extracted by Trizol (Invitrogen). The quality and quantity of RNA samples were determined by Agilent 2100 Bioanalyzer using RNA 6000 Nano Kit (Agilent Technologies, Inc.). The reverse transcription reactions were performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem). The expression of miRNA was detected by TaqMan® Rodent MicroRNA Array A (Applied Biosystems), and analyzed by Applied Biosystems 7900 HT Fast Real-Time PCR System containing 381 rodent miRNA targets.

Statistics

Statistical significance was determined using the Student's *t* test. In all figures, values were expressed as mean $\pm$ standard deviation (SD) and statistical significance ($p < 0.05$) was indicated by an asterisk. The data represent results from at least three independent experiments.

RESULTS

MiR-130a directly targets at both PGC1 α and PPAR γ

Four different target prediction algorithms were used to identify potential target transcription factors of miR-130a in hepatocytes. PPARGC1- α (PGC1 α) was identified as such a factor whose 3'UTR was consistently predicted by all four programs (Table 2).

Table 2. Prediction of miR-130a target sites at the 3'UTR of human transcription factors known to influence HBV transcription

Transcription factors ξ	Target scan	PicTar	DIANA	RNAhybrid
HNF1	+	-	-	-
HNF4 α	+	-	-	-
C/EBP α	-	-	-	-
C/EBP β	-	-	-	-

SP1	+	-	+	-
RXR α	+	-	+	-
PPAR α	+	-	-	-
PPARγ	+	-	-	+
FoxA3 (HNF3 γ)	-	-	-	-
FoxO1	-	-	-	-
FXR α	-	-	-	-
PGC1α	+	+	+	+
ERR α	-	-	-	-
COUP-TF	-	-	-	-
LRH	-	-	-	-

£ These transcription factors had been reported to be involved in HBV RNA synthesis in literatures^{5,6,38}. MiR-130a was shown to target at the 3' UTR of PPAR γ in adipocytes. Lee, E.K. et al. *Mol Cell Biol* 31, 626-638 (2011).

- 5 To address the potential relationship between miR-130a and PGC1 α , a stable cell line expressing miR-130a was established (Fig. 1). While the expression levels of most hepatic transcription factors being examined here remained unchanged in miR-130a expressing cell lines, simultaneous reductions of PPAR γ and PGC1 α mRNAs and proteins were observed (Figs. 2, 3 and 4), suggesting that miR-130a can target both PPAR γ and PGC1 α . PPAR γ is known to stimulate adiponectin production. Yu et al. *The Journal of biological chemistry* 278, 498-505 (2003). As expected, secreted adiponectin was significantly reduced in miR-130a expressing cell lines (Fig. 5). In a reciprocal experiment, HepG2 cells were treated with LNA-miR-130a antagomir, and significant increase of PGC1 α mRNA and protein was observed (Fig. 6). In accordance with this finding miR-130a was significantly reduced while PGC1 α , G6Pase, PEPCK, PPAR γ and serum adiponectin were all significantly increased the transgenic mice described in (Chen, C.C. et al. *Gene therapy* 14, 11-19 (2007)) (Fig. 7). Therefore, miR-130a may reduce PPAR γ and PGC1 α protein levels (Fig. 3) by reducing their respective mRNA levels (Figs. 2, 6). Interestingly, the combination of both PPAR γ and PGC1 α , in the absence of any exogenous ligands exhibited a dramatic synergistic effect on secretions of adiponectin (Fig. 8).
- 10
- 15
- 20

To distinguish between a direct and an indirect mechanism of miR-130a on reducing PGC1 α mRNA, a reporter assay was performed using 3'UTR from either PPAR γ or PGC1 α . The results support a functional interaction between miR-130a and the 3' UTR of PPAR γ or PGC1 α (Fig. 9). Next, compensatory mutations were introduced into the seed sequences of miR-130a and its evolutionarily conserved target site of PGC1 α (Fig. 10). By cotransfection assay, only the combination of a mutant miR-130a and a mutant PGC1 α could successfully restore the inhibitory effect of miR-130a on the luciferase activity. In a dose-dependent manner, miR-130a could reduce, while LNA-miR-130a could increase, the luciferase activity of a reporter containing the 3'UTR of PGC1 α (Fig. 11). Taken together, miR-130a can directly target at both PGC1 α and PPAR γ .

MiR-130a in hepatic gluconeogenesis and lipogenesis

The dual targets of PGC1 α and PPAR γ by miR-130a strongly suggest its important role in energy metabolism. Several key metabolic enzymes in glycolysis, gluconeogenesis, and lipogenesis were examined using stable miR-130a expressing cells (Fig. 12, *upper panel*). Both phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are rate-limiting gluconeogenic enzymes known to be under the positive control of PGC1 α , Yoon, J.C. et al. *Nature* 413, 131-138 (2001). Since PGC1 α mRNA and proteins were reduced in miR-130a expressing cells (Figs. 2 and 3), it was anticipated that PEPCK and G6Pase should be reduced as well. Indeed, concurrent reductions of PEPCK and G6Pase mRNAs (Fig. 12) and proteins (Fig. 13) were observed in miR-130a expressing hepatocytes. While this result suggested a glycolysis rather than a gluconeogenesis pathway, it would be more certain if their glycolytic counterparts, pyruvate kinase (PKLR) and glucokinase (GCK), was not reduced simultaneously (Fig. 14). Indeed, the protein expression of GCK and PLKR was not reduced but increased in HepG2 or Huh7 cells (Fig. 13). Furthermore, increased mRNA expression of both GCK and PLKR was observed in miR-130a expressing hepatocytes (Fig. 14). These results suggest that miR-130a can not only inhibit HBV replication, but also contribute to downregulation of gluconeogenesis and upregulation of glycolysis in hepatocytes via PGC1 α . Thus, miR-130a would be effective in diseases and disorders associated with dysregulation of glycolysis, such as metabolic diseases.

A metabolic triad

HBV may exert an effect on PGC1 α and PPAR γ . Consistent with the reduction of miR-130a, PGC1 α , PEPCK, G6Pase, PPAR γ and secreted adiponectin proteins were all significantly increased in HBV-producing UP7-4 and UP7-7 cells (Figs. 15). Similarly, glucose production was significantly increased in stable PGC1 α -producing cells, and decreased in PPAR γ -producing cells (Fig. 16). No significant difference in glucose level was detected between control and miR-130a producing cells. As expected, the glucose level was increased in HBV-producing HepG2 cells, and further increased when HepG2 cells were treated with a PPAR γ antagonist, GW9662, irrespective of its HBV-producing status (Fig. 17). Finally, there was no apparent change in miR-130a expression in stable PGC1 α -expressing cell lines (Figs. 18).

A triad relationship among HBV, miR-130a, and PGC1 α is outlined in Fig. 19. In contrast to PGC1 α , expression of miR-130a was reduced in stable PPAR γ -expressing cell line, and further reduced by Rosiglitazone treatment, but not by GW9662 (Fig. 20). These results suggest another triad diagram with a positive feed-forward loop (Fig. 21). A primary weak signal received by this loop may be amplified into a stronger phenotypic outcome.

DISCUSSION

The most salient feature of miR-130a is its dual targets at PPAR γ and PGC1 α (Figs. 2, 3 and 5, 9, 10), leading to reduced HBV replication. Conversely, HBV can reduce the expression of miR-130a (Table 2.), leading to increased expression of PPAR γ (adiponectin) and PGC1 α (PEPCK and G6Pase) (Figs. 12, 13, and 15). Overexpression of PPAR γ reduced the level of miR-130a, and further reduction of miR-130a was observed by Rosiglitazone (Fig. 20) or the combination of PPAR γ and PGC1 α . Taken together, a positive feed-forward loop among HBV, miR-130a, PGC1 α and PPAR γ , was established (Fig. 21). This triad loop could in theory magnify a weak primary signal by going through this loop repetitive rounds.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

What is claimed is:

1. A pharmaceutical composition for use in treating a disease associated with PGC1 α or PGC1 β , the pharmaceutical composition comprising an effective amount of one or more oligonucleotides and a pharmaceutically acceptable, wherein the one or more oligonucleotides are:

- (a) a miR-130a RNA, a miR-130b RNA, or a combination thereof; or
- (b) an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof.

2. The pharmaceutical composition for use of claim 1, wherein the one or more oligonucleotides are duplex RNA molecules or single-strand RNA molecules.

3. The pharmaceutical composition for use of claim 1 or claim 2, wherein the oligonucleotide is a miR-130a RNA or a miR-130b RNA, which comprises the nucleotide sequence of AGUGCAA.

4. The pharmaceutical composition for use of claim 3, wherein the miR-130a RNA comprises the nucleotide sequence of CAGUGCAAUGUAAAAGGGCAU (SEQ ID NO:1).

5. The pharmaceutical composition for use of claim 3, wherein the miR-130b RNA comprises the nucleotide sequence of CAGUGCAAUGAUGAAAGGGCAU (SEQ ID NO:2).

6. The pharmaceutical composition for use of any of claims 1-5, wherein the one or more oligonucleotides are a miR-130a RNA, a miR-130b RNA, or a combination thereof; and the pharmaceutical composition is for use in treating diabetes or steatosis.

7. The pharmaceutical composition for use of any of claims 1-5, wherein the one or more oligonucleotides are an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof; and the pharmaceutical composition is for use in treating a disease associated with reactive oxygen species (ROS) or diabetes.

8. The pharmaceutical composition for use of claim 7, wherein the pharmaceutical composition is for use in treating a neurodegenerative disease, a cardiovascular disease, inflammation, or muscle dysfunction.

9. The pharmaceutical composition for use of claim 8, wherein the neurodegenerative disease is Parkinson disease, Hungtinton disease, or Alzheimer disease.

10. The pharmaceutical composition for use of claim 8, wherein the cardiovascular disease is heart failure.

11. The pharmaceutical composition for use of any of claims 1-10, wherein the one or more oligonucleotides are an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof, and wherein the antagomiR is a microRNA sponge or a locked nucleic acid.

12. A method for regulating PGC1 α or PGC1 β , comprising contacting cells with an effective amount of:

- (c) a miR-130a RNA, a miR-130b RNA, or a combination thereof; or
- (d) an antagomiR of miR-130a, an antagomiR of miR-130b, or a combination thereof.

13. The method of claim 12, wherein the cells are contacted with the miR-130a, the miR-130b, or a combination thereof in an amount effective to down-regulate PGC1 α or PGC1 β .

14. The method of claim 12 or claim 13, wherein the miR-130a RNA, the miR-130b RNA, or both are duplex RNA molecules or single-strand RNA molecules.

15. The method of any of claims 12-14, wherein the miR-130a RNA or the miR-130b RNA comprises the nucleotide sequence of AGUGCAA.

16. The method of claim 15, wherein the miR-130a RNA comprises the nucleotide sequence of CAGUGCAAUGUUAAAAGGGCAU (SEQ ID NO:1).

17. The method of claim 16, wherein the miR-130b RNA comprises the nucleotide sequence of CAGUGCAAUGAUGAAAGGGCAU (SEQ ID NO:2).

18. The method of claim 12, wherein the cells are contacted with the antagomiR of miR-130a, the antagomiR of miR-130b, or a combination thereof in an amount effective to up-regulate PGC1 α or PGC1 β .

19. The method of claim 18, wherein the antagomiR of miR-130a, the antagomiR of miR-130b, or both are duplex RNA molecules or single-strand RNA molecules.

20. The method of any of claims 12-19, wherein the contacting step is performed by administering a subject in need thereof an effective amount of one or both of the miRNAs or one or both of the antagomiRs.

21. The method of claim 20, wherein the subject is administered with the miR-130a, the miR-130b, or a combination thereof.

22. The method of claim 21, wherein the subject has or is suspected of having diabetes or steatosis.

23. The method of claim 20, wherein the subject is administered with the antagomiR of miR-130a, the antagomiR of miR-130b, or a combination thereof.

24. The method of claim 23, wherein the subject has or is suspected of having a disease associated with reactive oxygen species (ROS).

25. The method of claim 23, wherein the disease associated with ROS is selected from the group consisting of muscle dysfunction, heart failure, and a neurodegenerative disease.

26. The method of claim 25, wherein the neurodegenerative disease is Parkinson disease, Huntington disease, or Alzheimer disease.

27. The method of claim 23, wherein the subject has or is suspected of having diabetes or inflammation.

28. The method of any of claims 12-27, wherein the antagomiR of miR-130a, the antagomiR of miR-130b, or both are microRNA sponges or a locked nucleic acid (LNA).

Fig. 1

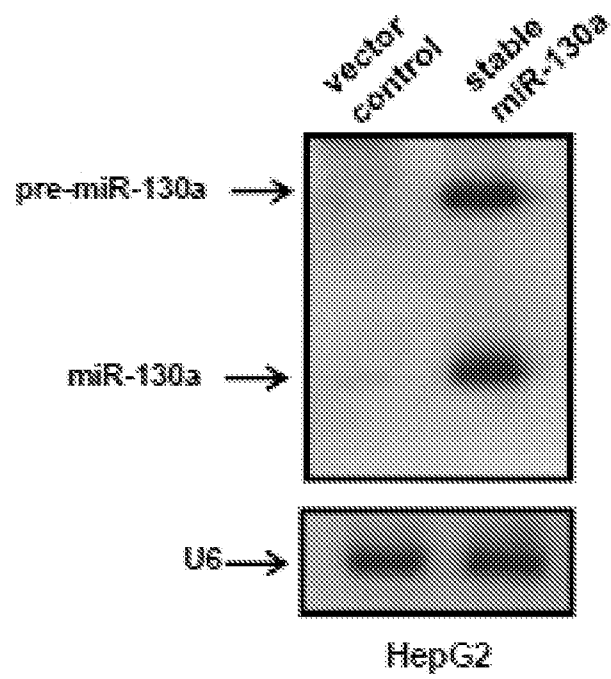


Fig. 2

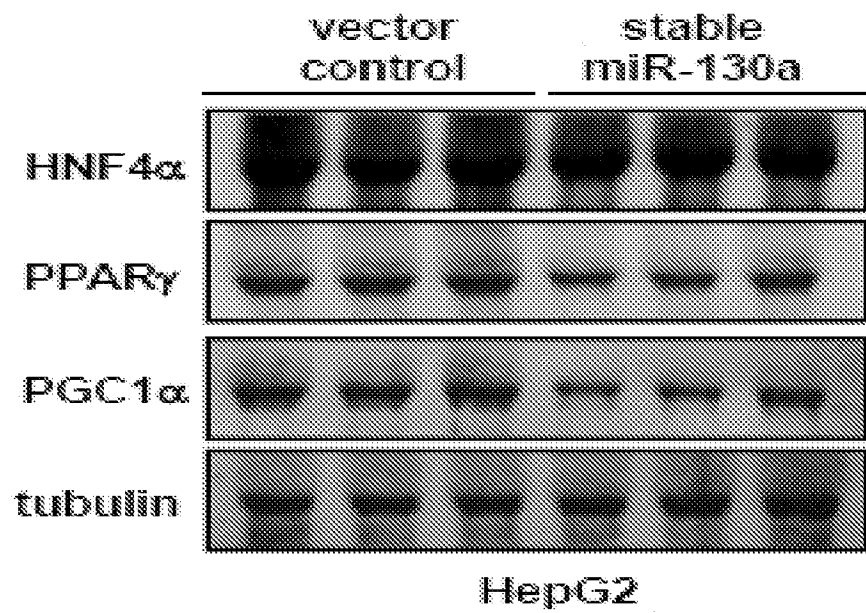


Fig. 3

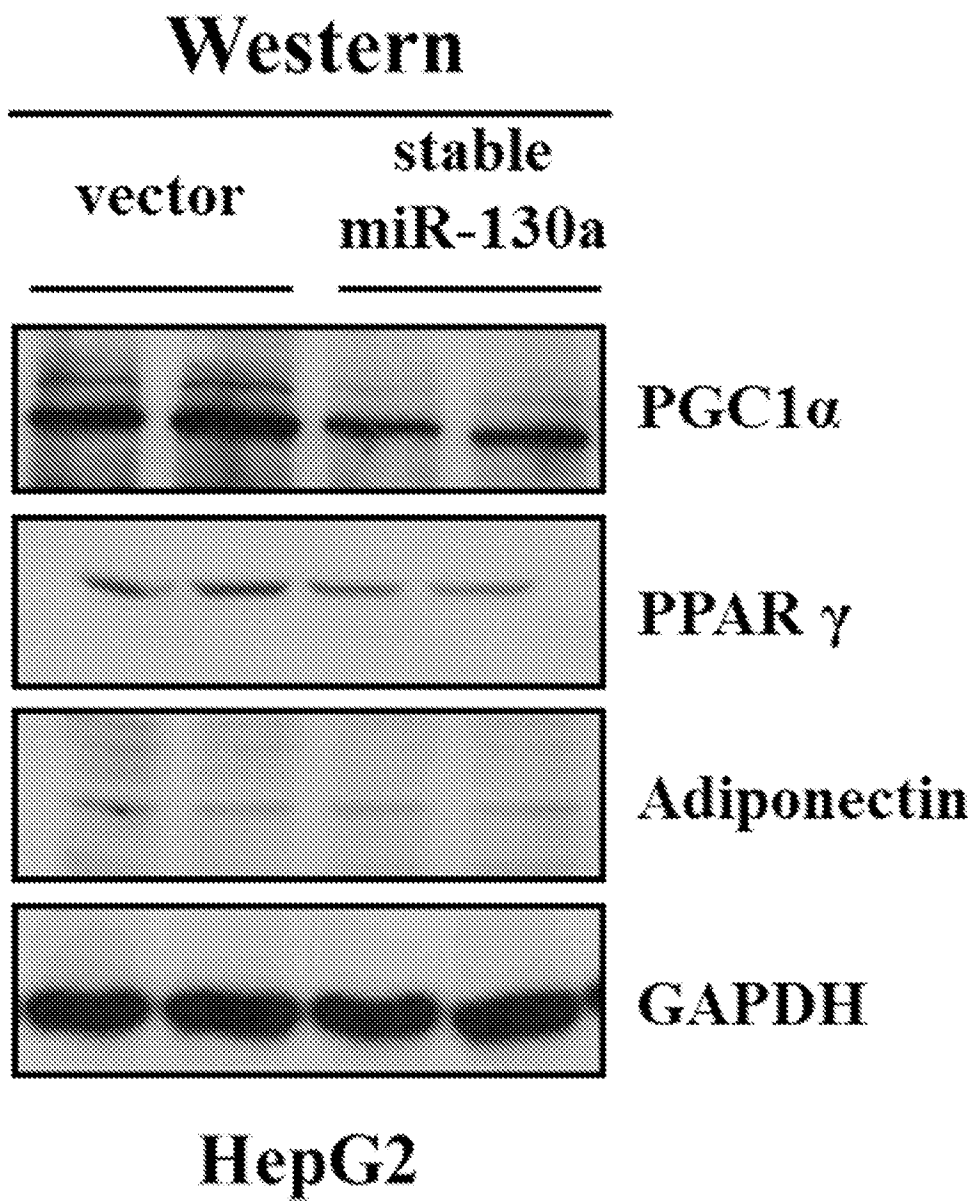
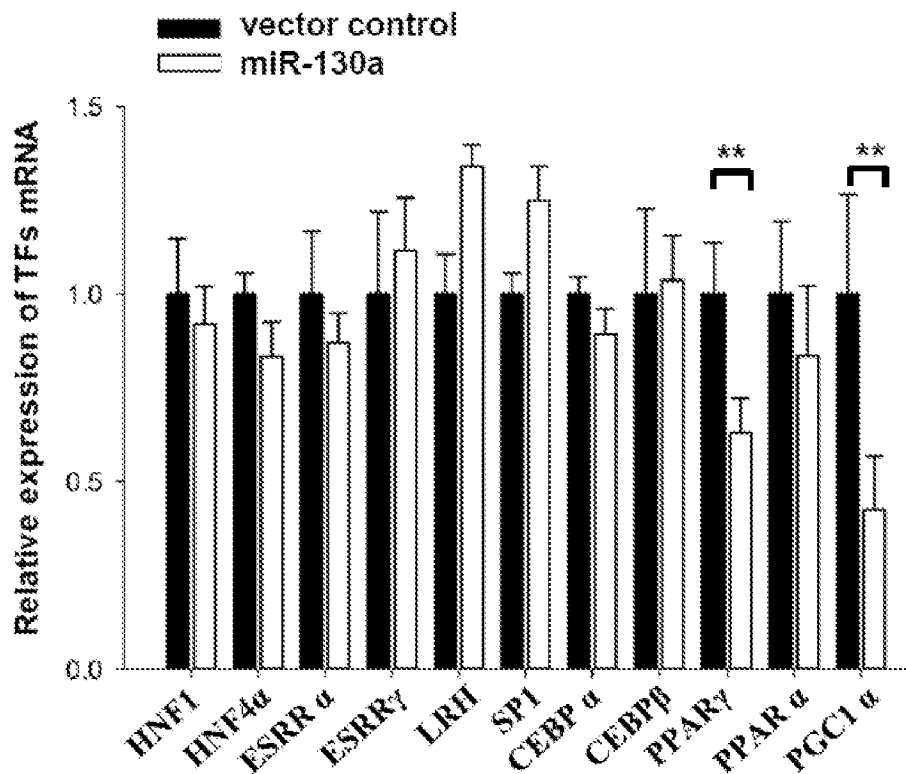


Fig. 4

stable-miR-130a-HepG2 cells



Western

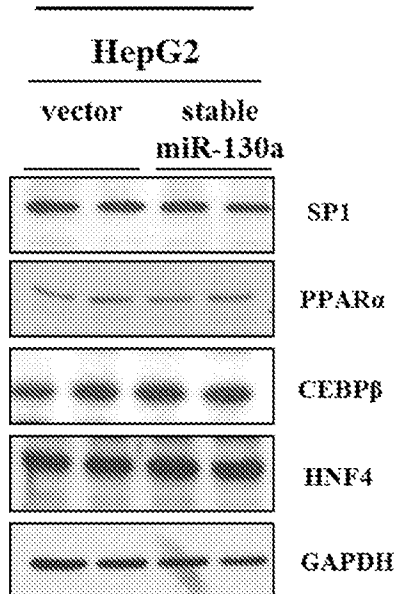
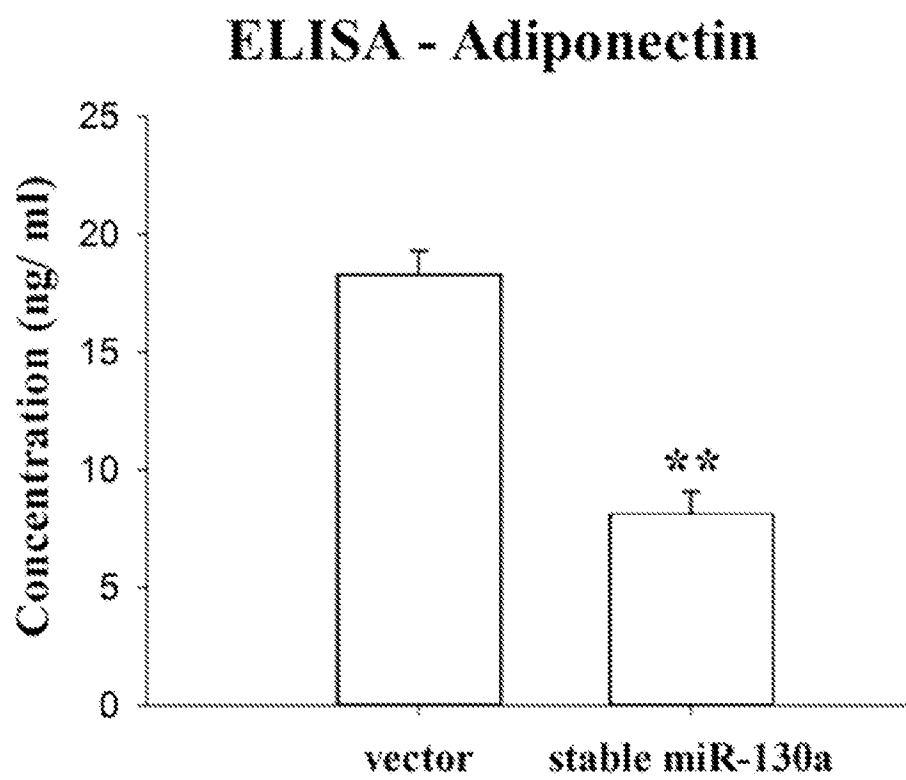


Fig. 5



HepG2

Fig. 6

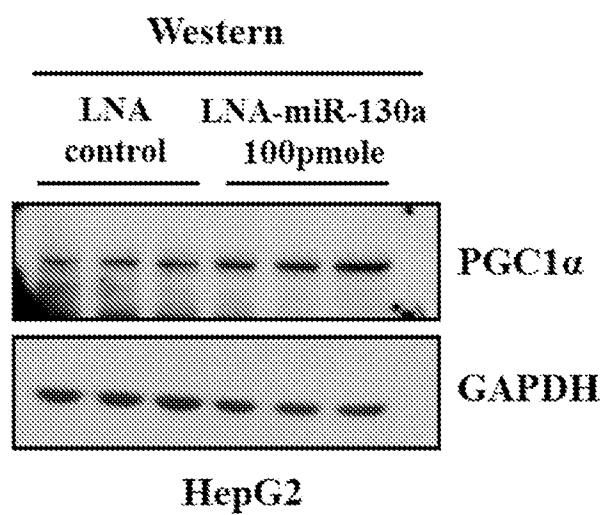
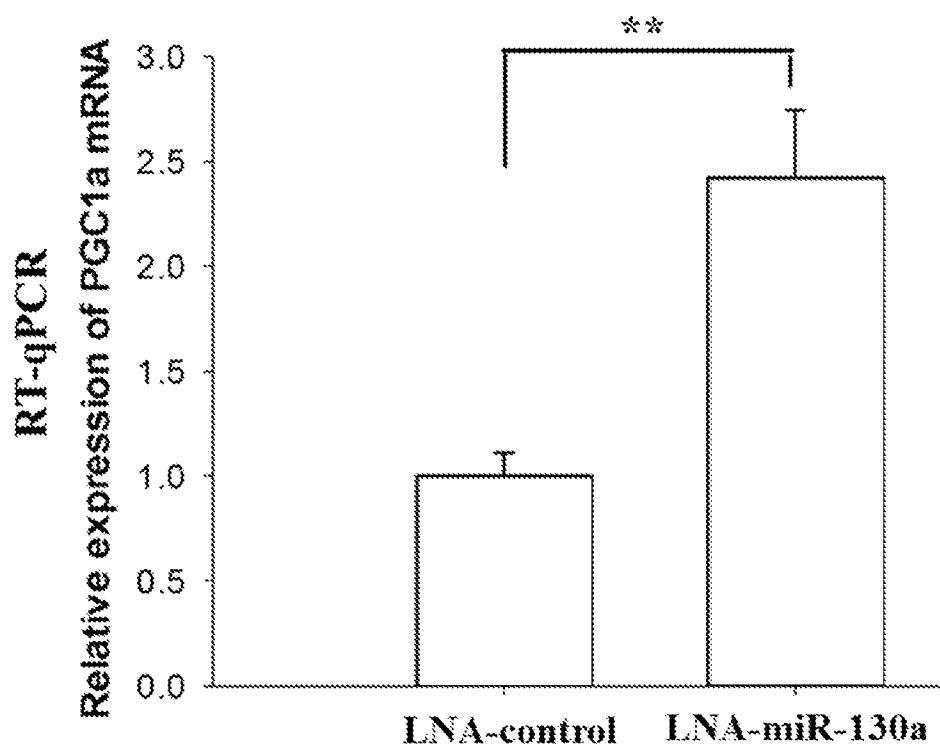


Fig. 7

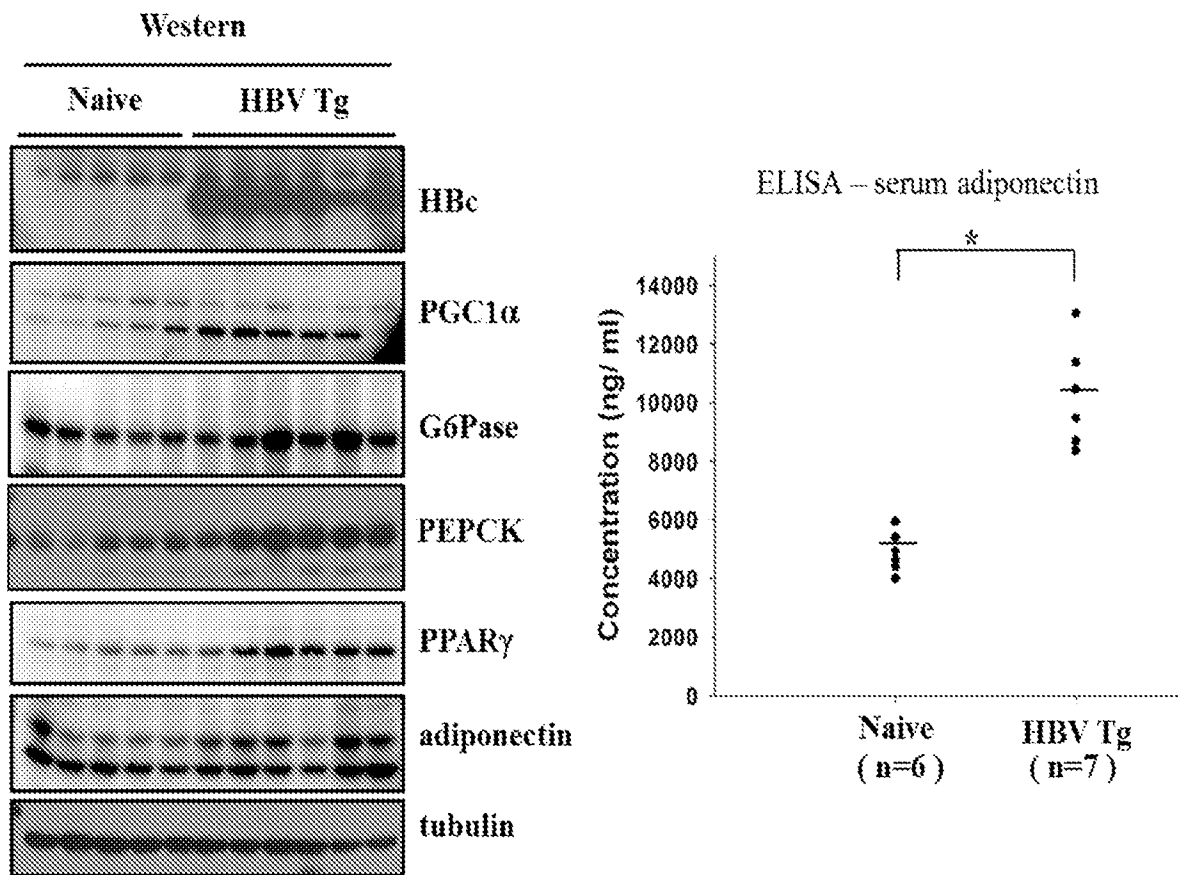


Fig. 8

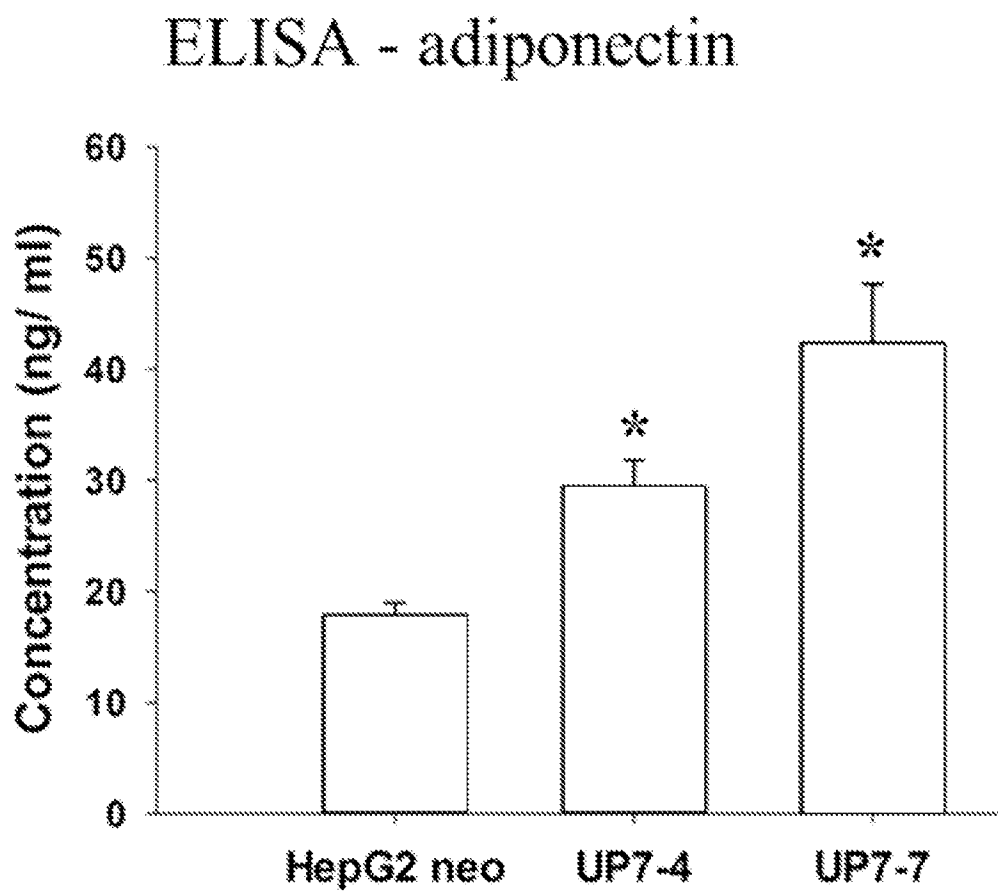


Fig. 9

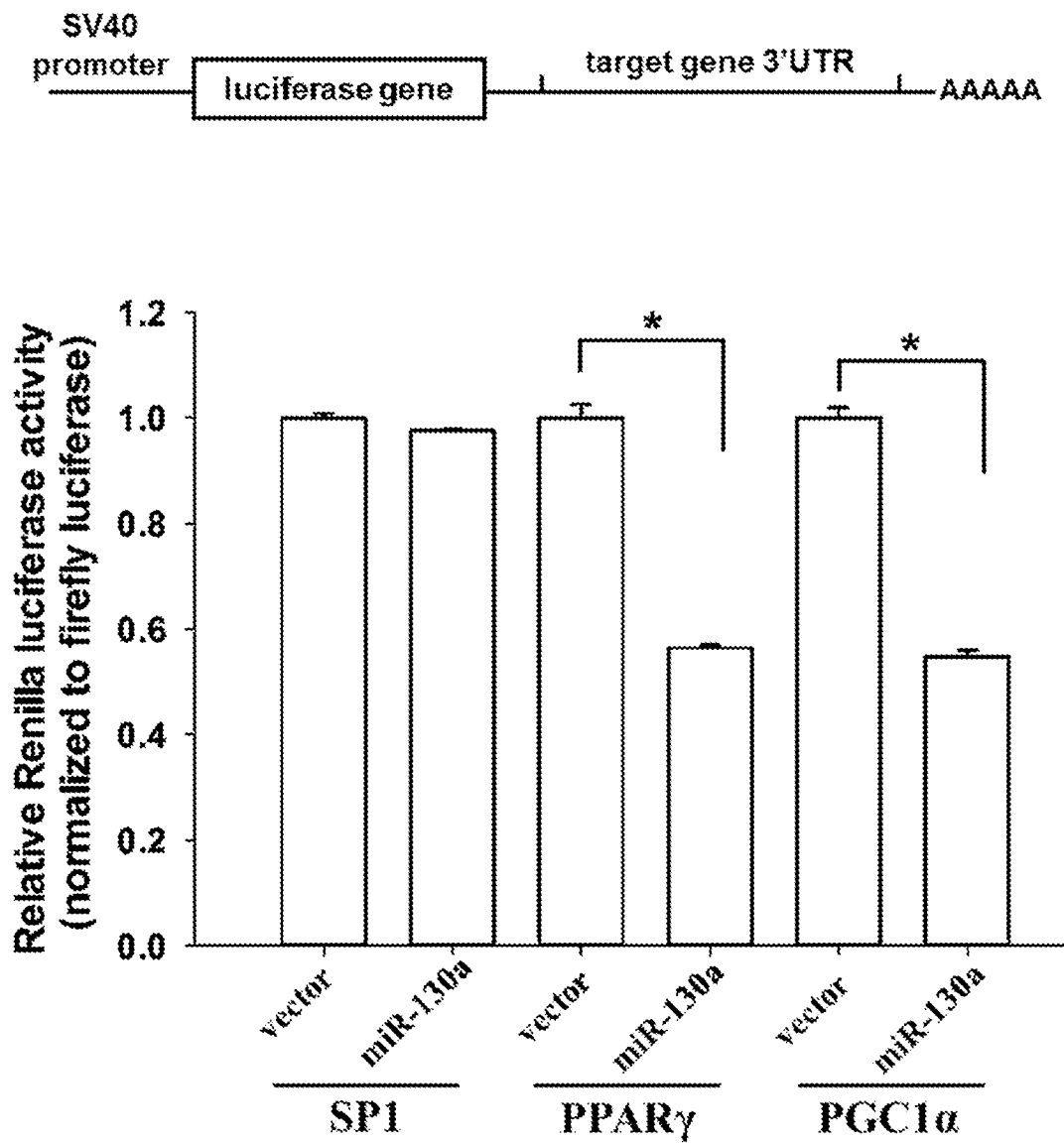


Fig. 10

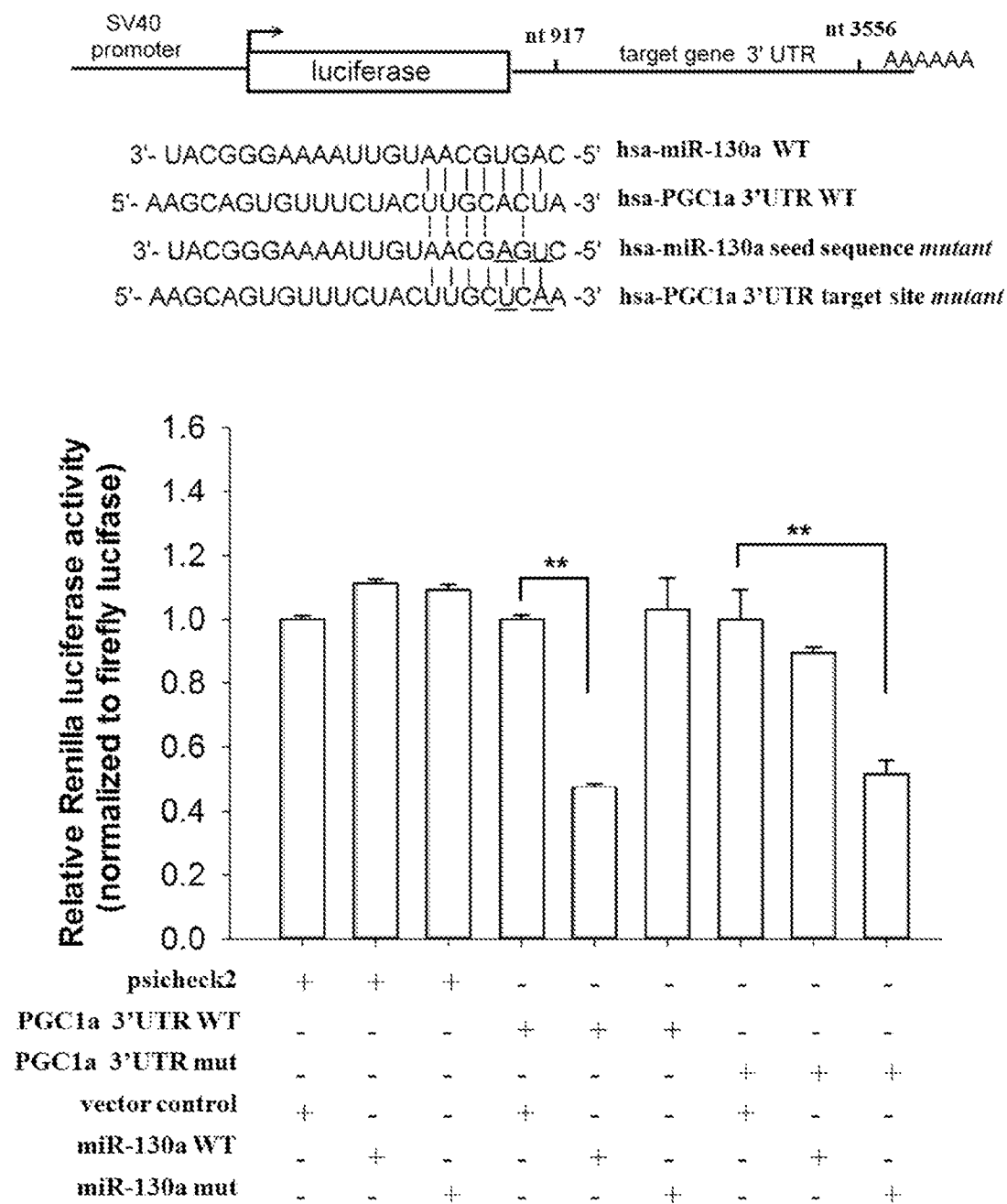


Fig. 11

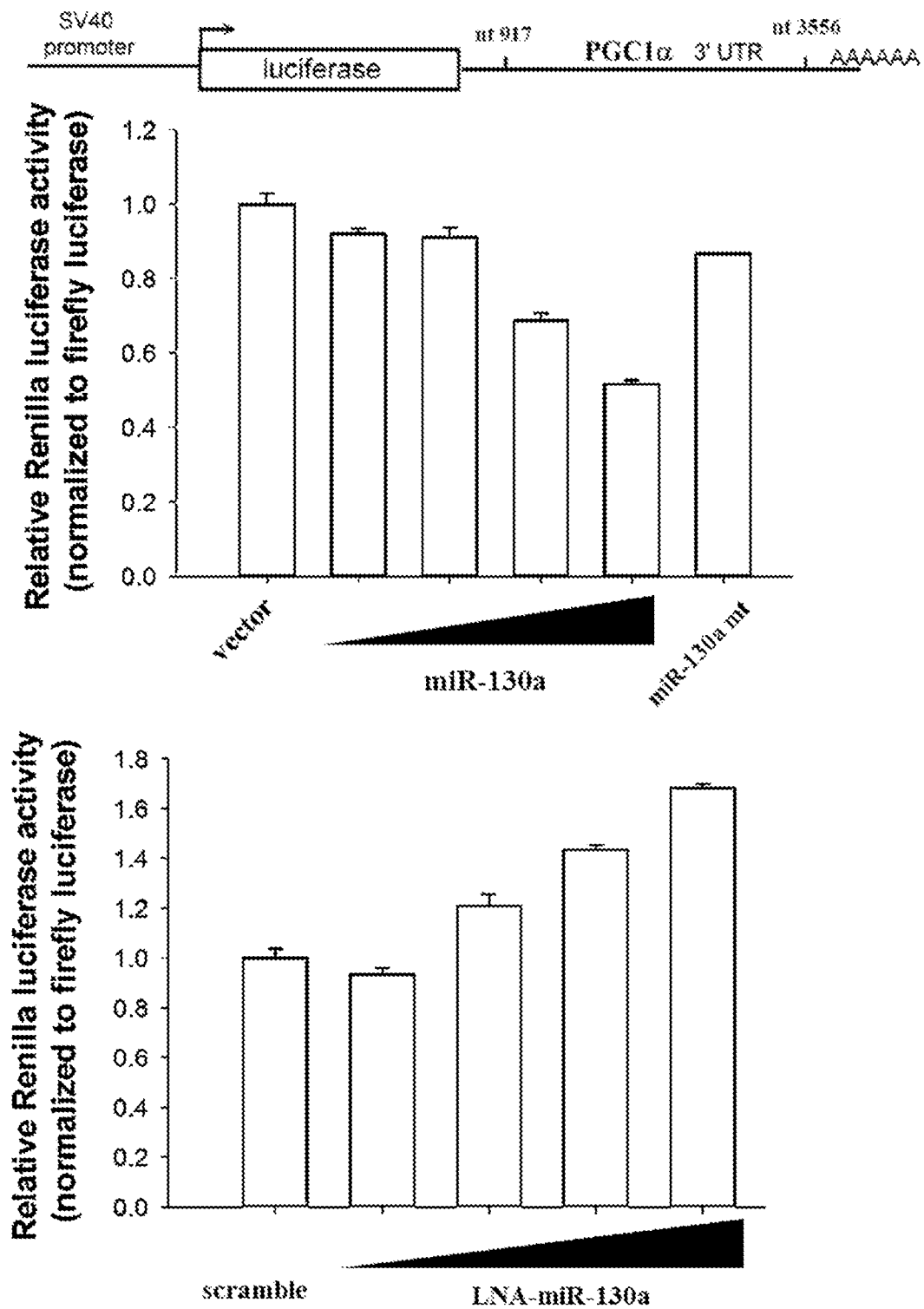
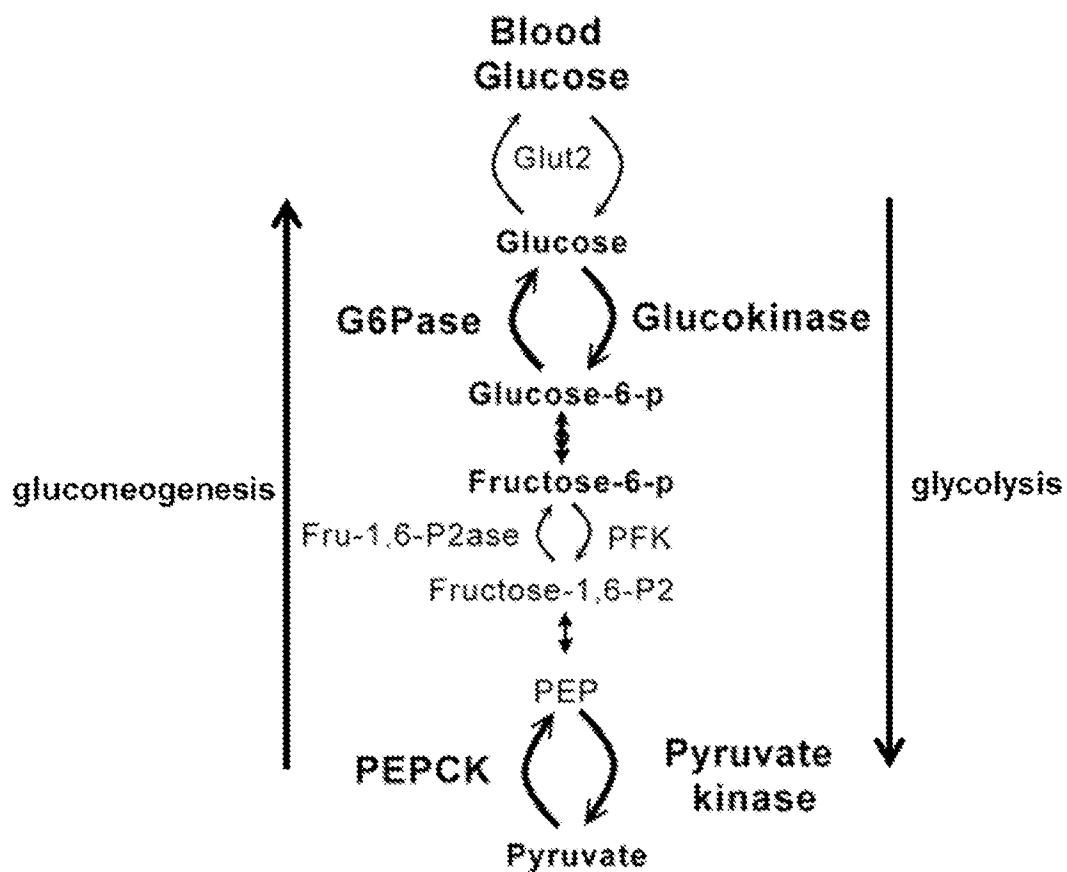


Fig. 12



Northern

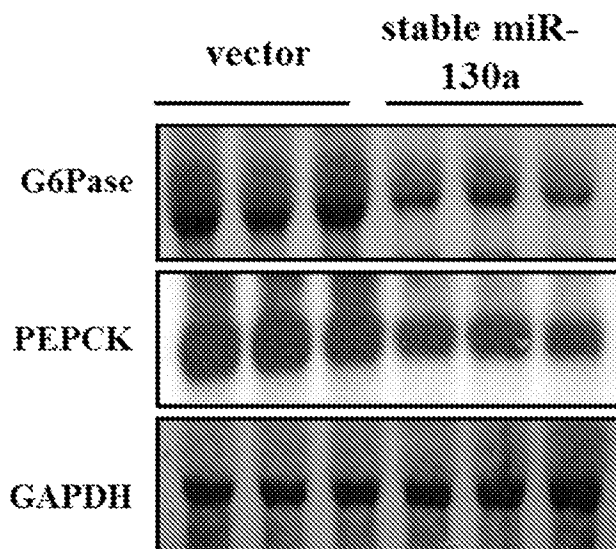


Fig. 13

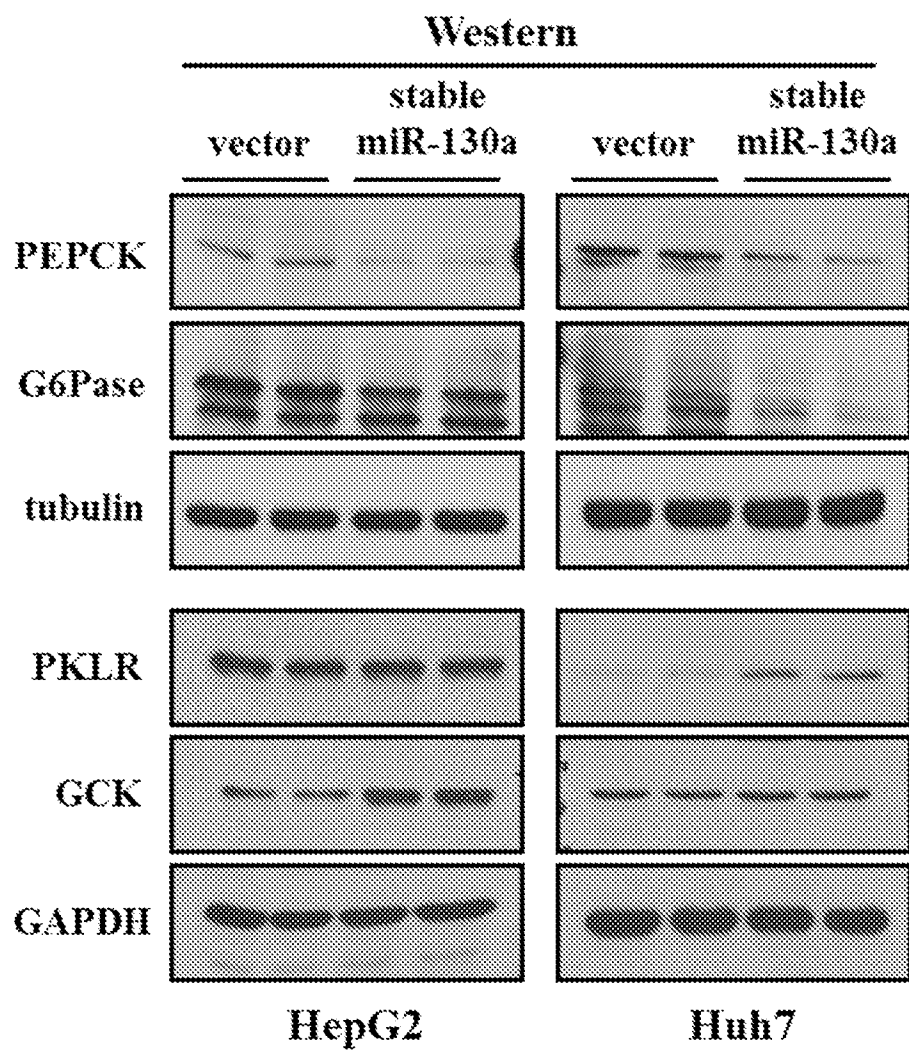


Fig. 14

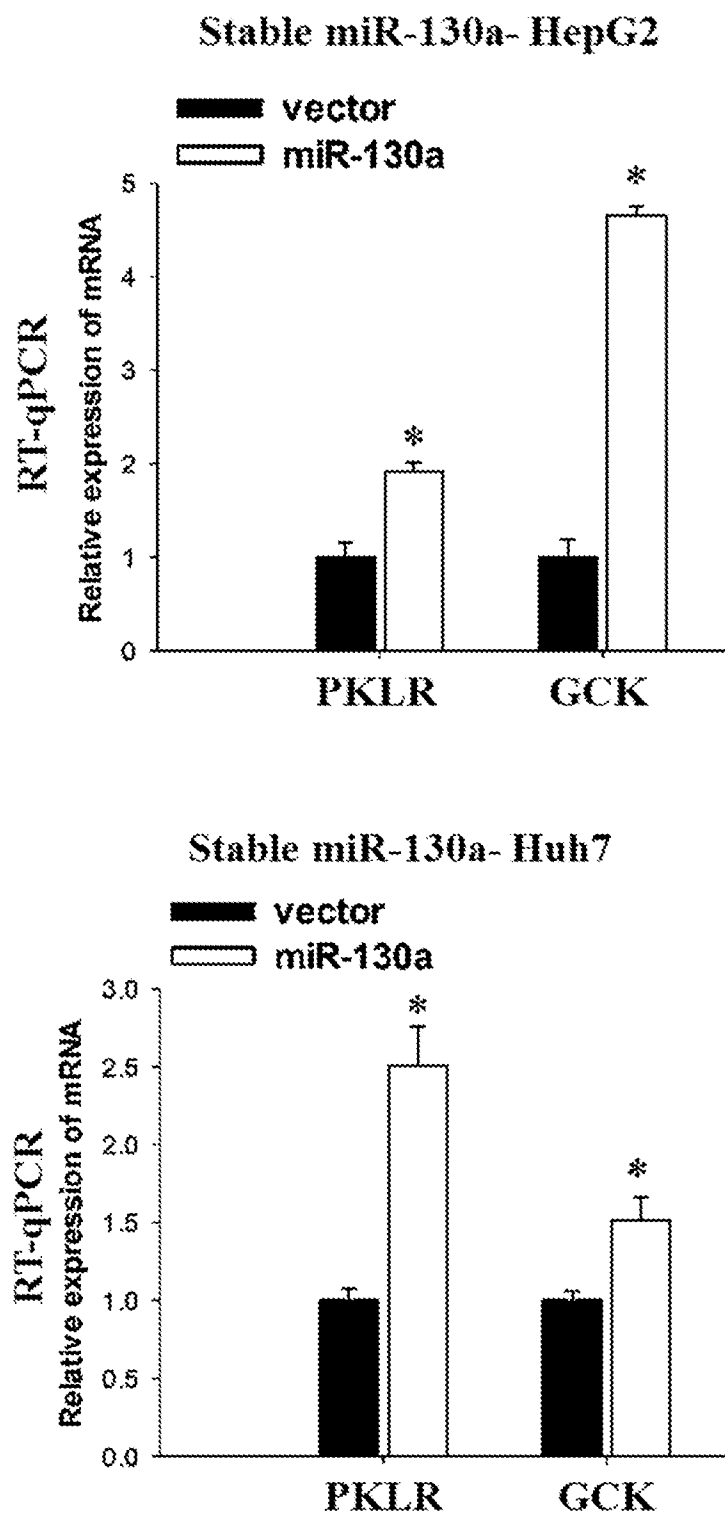


Fig. 15

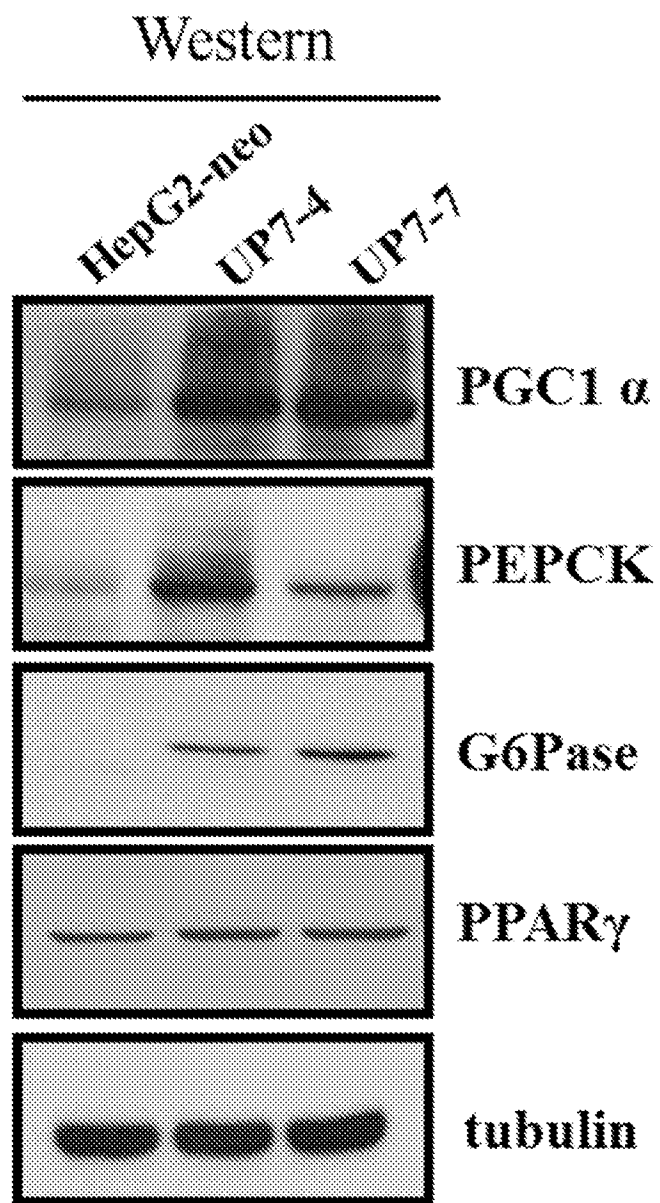


Fig. 16

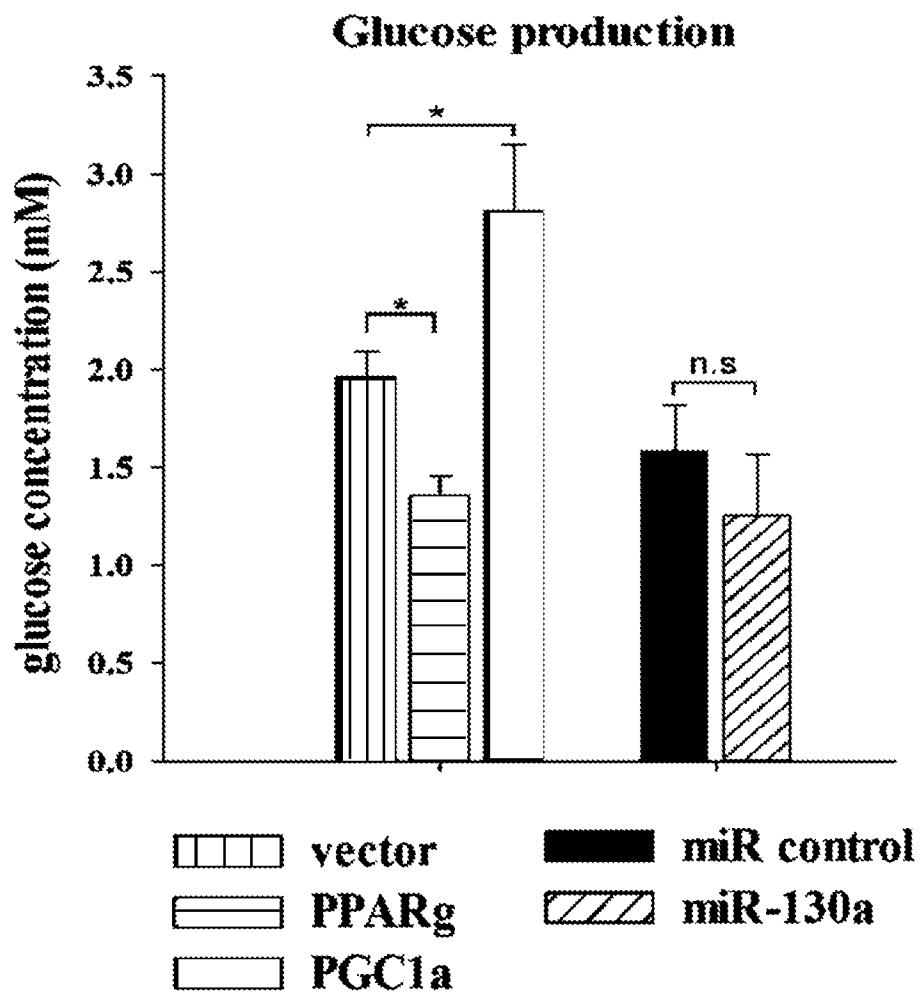


Fig. 17

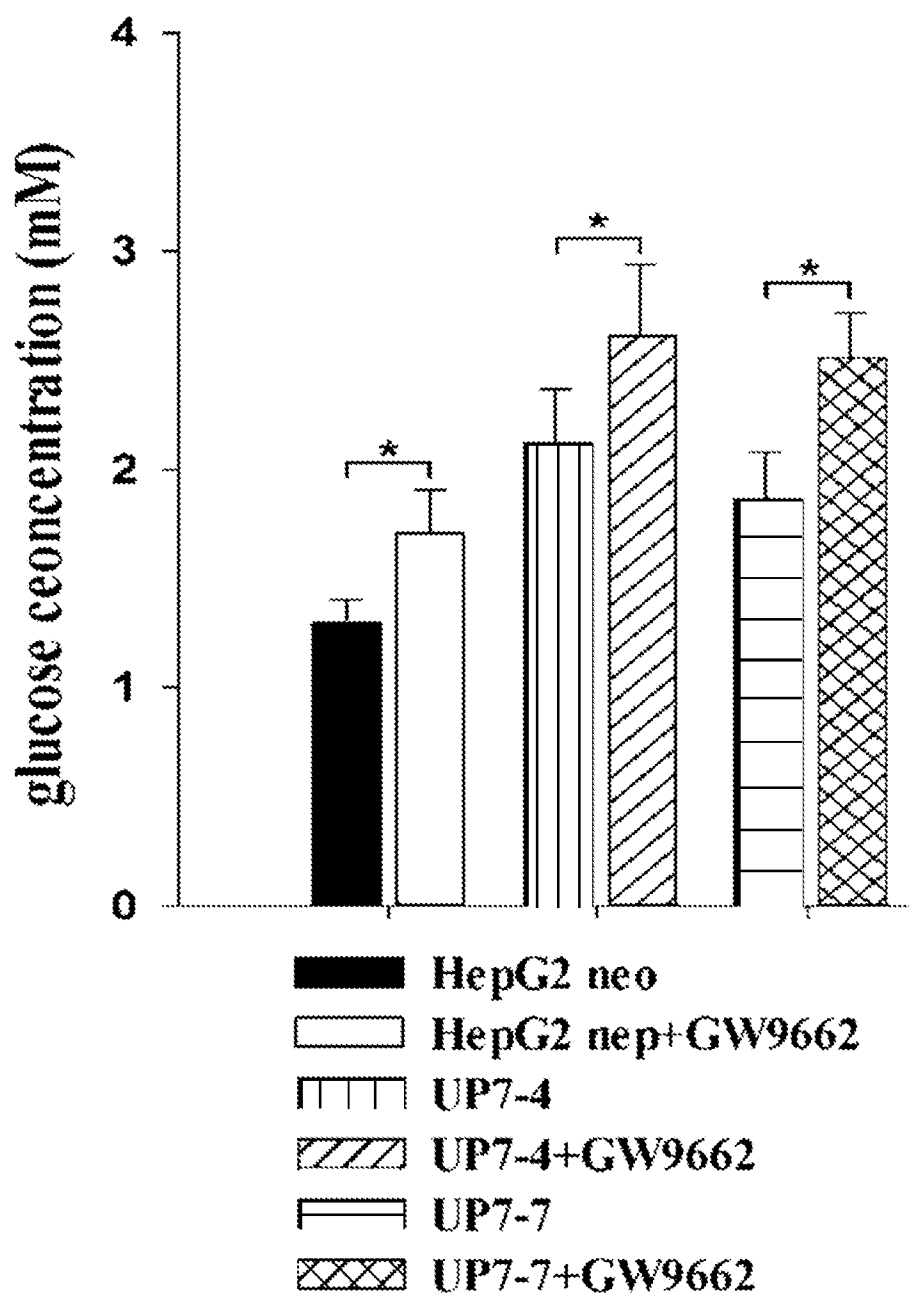


Fig. 18

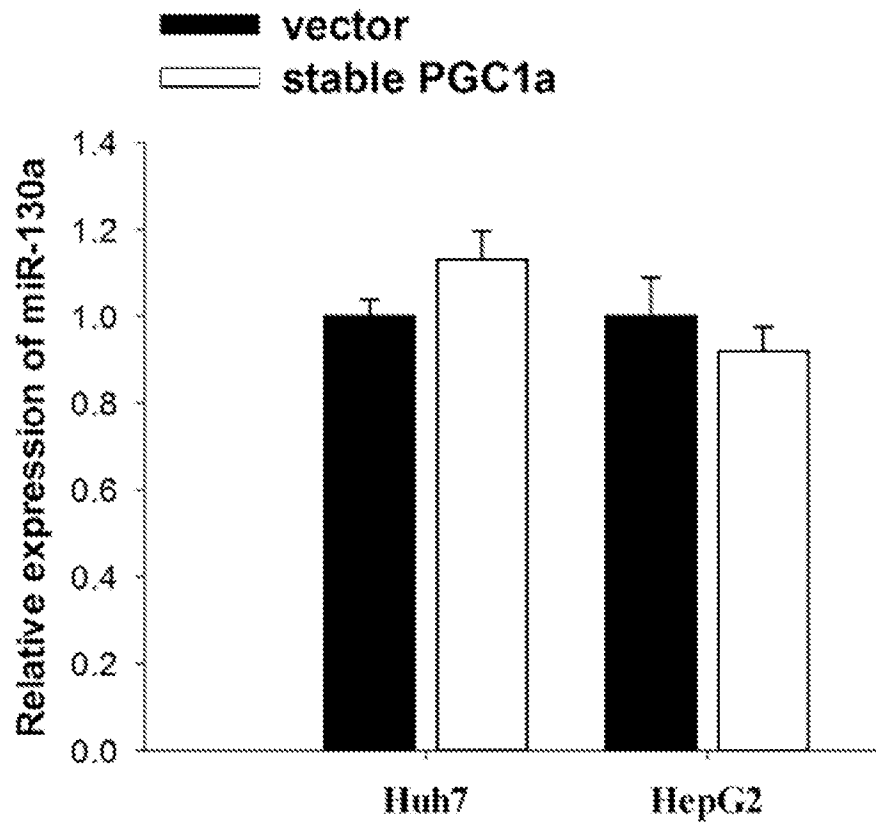


Fig. 19

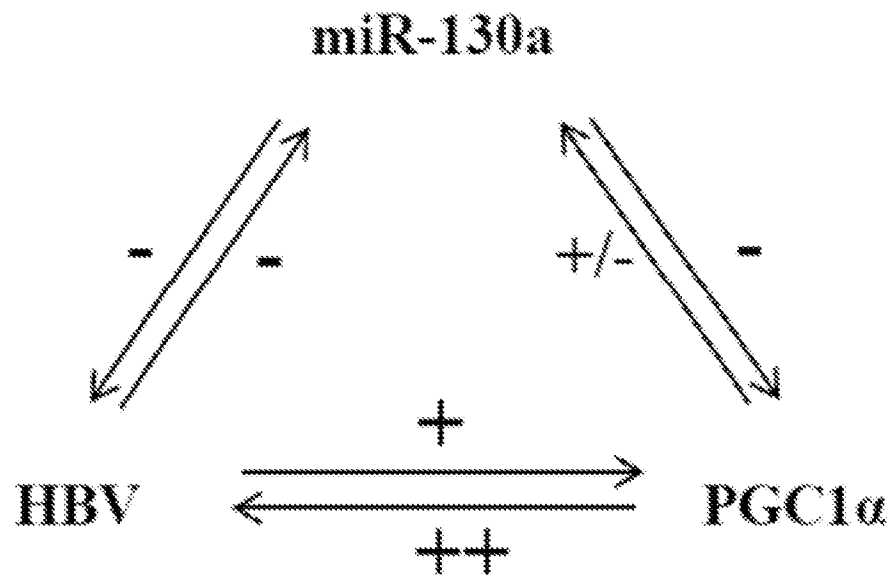


Fig. 20

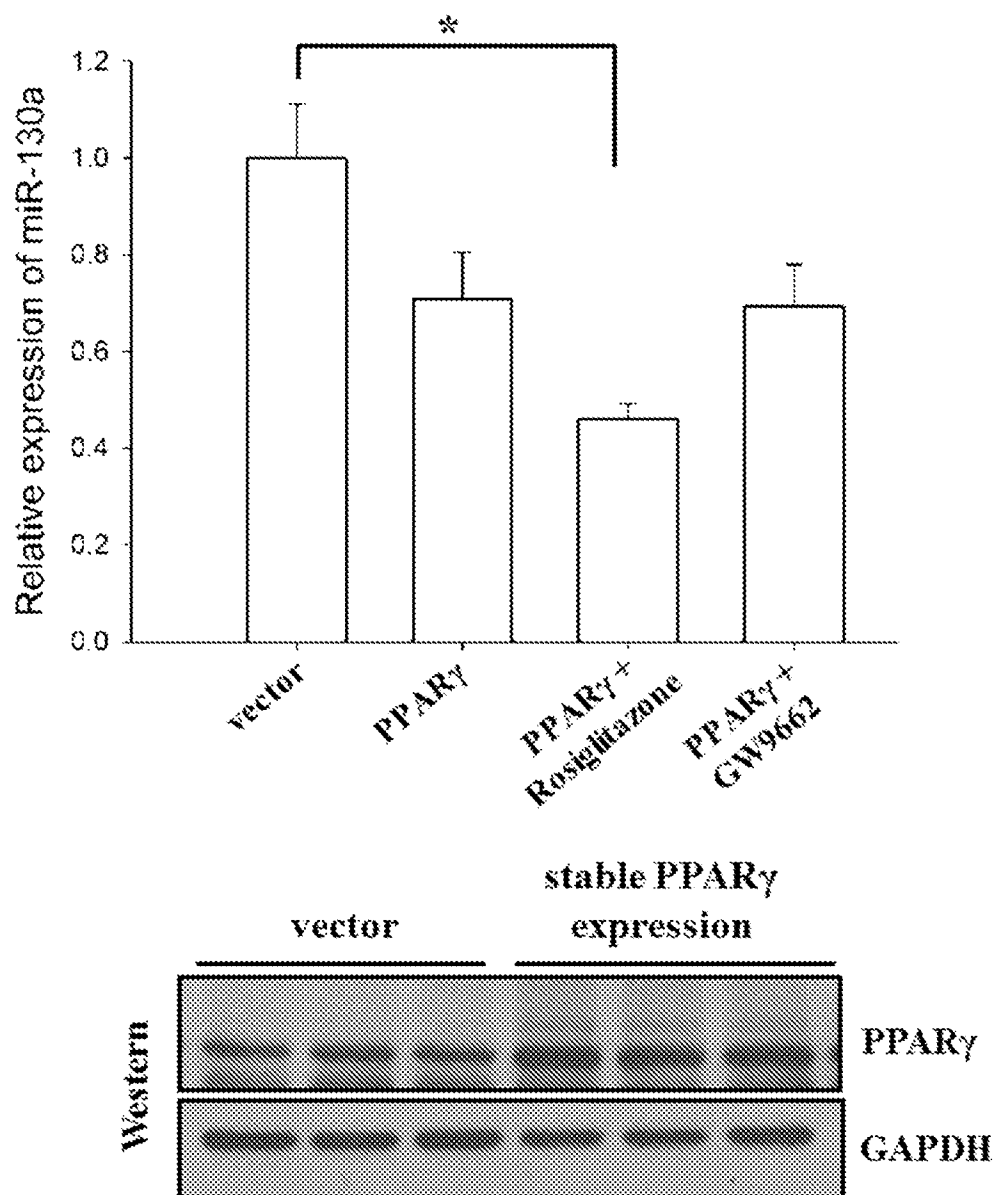


Fig. 21

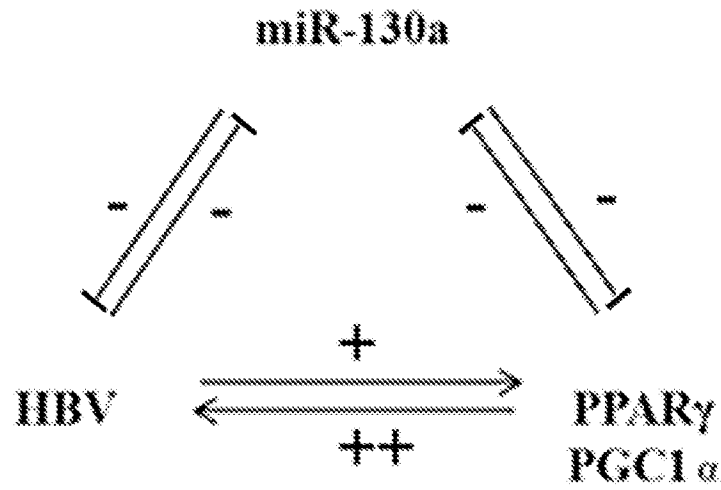
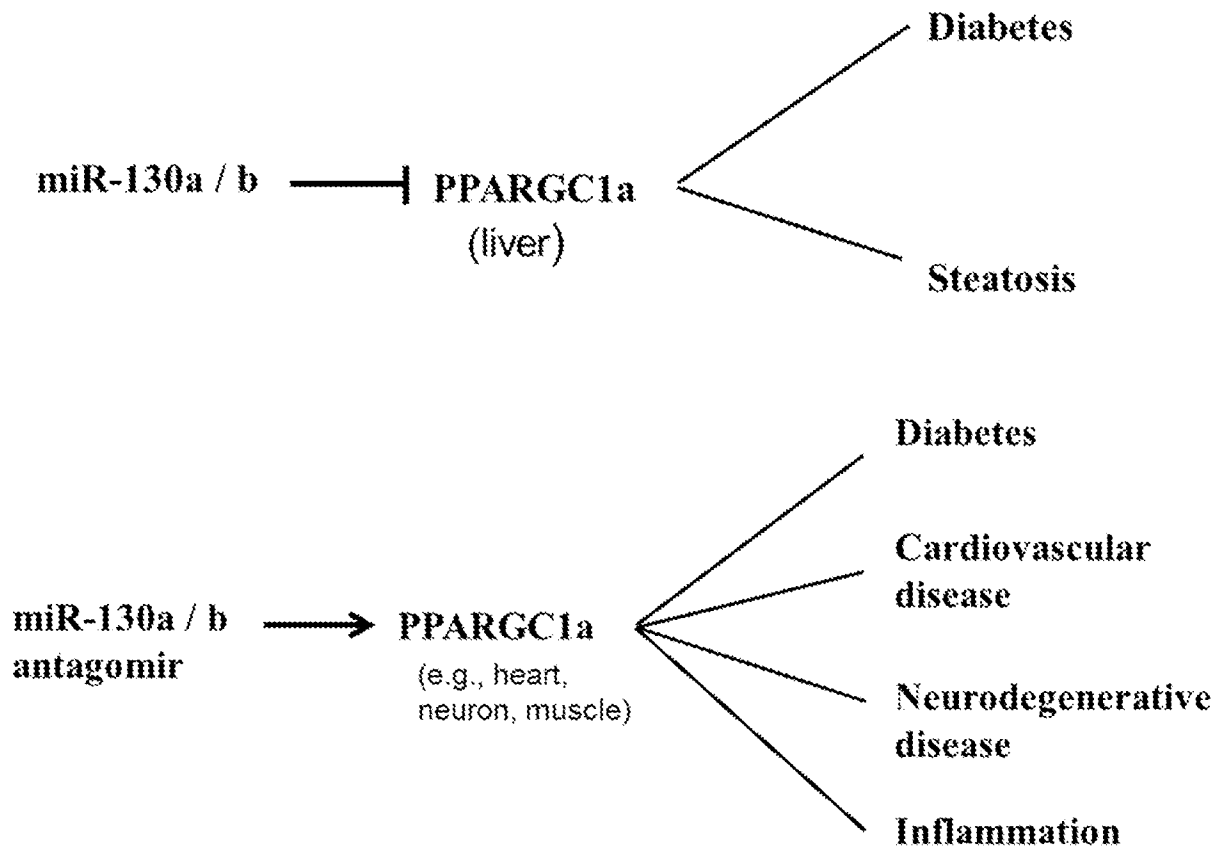


Fig. 22



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/056611**A. CLASSIFICATION OF SUBJECT MATTER****A61K 48/00(2006.01)i, A61P 3/10(2006.01)i, A61P 3/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 48/00; C12Q 1/68; A61P 3/10; A61P 3/00

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: miR-130a, miR-130b, PGC1 α , PGC1 β , antagomiR, diabetes, steatosis**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG, Y.-C. et al., 'Circulating miR-130b mediates metabolic crosstalk between fat and muscle in overweight/obesity', Diabetologia, 19 July 2013 (E-pub.), Vol. 56, pp. 2275-2285 See abstract and pages 2282-2284.	1-2
A		3-5
A	BICCHI, I. et al., 'MicroRNAs and molecular mechanisms of neurodegeneration', Genes, 29 May 2013, Vol. 4, pp. 244-263 See abstract; pages 253-254; and Table 1.	1-5
A	TANG, L. et al., 'The hepatitis B virus-associated estrogen receptor alpha (ER α) was regulated by microRNA-130a in HepG2.2.15 human hepatocellular carcinoma cells', Acta Biochimica et Biophysica Sinica, 2011, Vol. 43, pp. 640-646 See abstract and pages 642-645.	1-5
A	CN 101368213 A (NANJING UNIVERSITY) 18 February 2009 See abstract; pages 9-12; and claim 1.	1-5



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

14 January 2015 (14.01.2015)

Date of mailing of the international search report

14 January 2015 (14.01.2015)

Name and mailing address of the ISA/KR

International Application Division
Korean Intellectual Property Office
189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea

Facsimile No. ++82 42 472 3473

Authorized officer

CHOI, Sung Hee

Telephone No. +82-42-481-8740



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/056611

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AOI, W. et al., `The microRNA miR-696 regulates PGC-1 $\alpha$ in mouse skeletal muscle in response to physical activity`, American Journal of Physiology-Endocrinology and Metabolism, 2010, Vol. 298, pp. E799-E806 See abstract.	1-5

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/056611**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☒ Claims Nos.: 12-28
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 12-28 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☒ Claims Nos.: 8-10,16-17,21-27
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 8-10,16-17 and 21-27 are regarded to be unclear since they refer to multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 6-7,11,15,20,28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

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

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/056611

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 101368213 A	18/02/2009	CN 102016037 A CN 102016037 B WO 2010-043114 A1	13/04/2011 10/04/2013 22/04/2010