The present invention relates to the prevention and treatment of diabetic cardiomyopathy.
METHODS FOR THE PREVENTION AND TREATMENT OF DIABETIC CARDIOMYOPATHY USING MIR-424/322

FIELD OF THE INVENTION:
The present invention relates to the prevention and treatment of diabetic cardiomyopathy.

BACKGROUND OF THE INVENTION:
Diabetes is a leading cause of mortality and morbidity in the world. Type 2 diabetes, characterized by hyperglycemia and hyperinsulinemia with insulin-resistance, is in constant expansion in the developed countries due to social and environmental factors (such as aging, sedentarity, and obesity) and affects more than 300 millions in the world (Wild, Roglic et al. 2004). Defects in insulin signaling are among the most common and earliest defects that predispose an individual to the development of type 2 diabetes. Many of the complications of diabetes are of vascular origin. Indeed high blood glucose level leads to macrovascular and/or microvascular lesions and this puts diabetics at an increased risk of ischemic heart disease, renal failure, stroke, lower limb amputations and blindness (Winer and Sowers 2004). Altogether over 50% of deaths among diabetic patients are a result of cardiovascular disease. Patients with diabetes are at increased risk for developing coronary artery disease (CAD), hypertension, and heart failure (HF), and the majority of these patients succumb ultimately to heart disease. Cardiac insulin resistance is a common feature associated with type 2 diabetes, independently of high-fat feeding (Park, Cho et al. 2005). In the early stage of CAD, hypertension or changes in coronary blood flow (Iozzo, Chareonthaitawee et al. 2002), it might be a physiological event when excess lipid supply increases lipid utilization and reduces glucose metabolism. However chronic state of insulin resistance and dysregulated metabolism might induce cardiac remodeling and systolic dysfunction (Gray and Kim). On the other hand the diabetic state can also directly induce abnormalities in cardiac tissue independent of vascular defects, CAD or hypertension: defects in diastolic left ventricular function, with or without systolic left ventricular dysfunction followed by left ventricular hypertrophy and fibrosis are observed in the hearts of patients with both Type 1 and Type 2 diabetes and rodents models of chronic diabetes. This particular and specific cardiac effect of diabetes has been called diabetic cardiomyopathy. Despite the increase in the characterization and comprehension of the cardiovascular consequences of diabetes, all the mechanisms
implicated are still not clearly understood as they result from multifactorial and complex molecular and cellular changes.

Accordingly, there is a need to develop new drugs that will be suitable for preventing or treating diabetic cardiomyopathy. In this way, it has been suggested that characterization of new therapeutic compounds in diabetic cardiomyopathy may be highly desirable.

Recent data have demonstrated the importance of miRNA in the post-transcriptional regulation of cardiac and vascular pathophysiology as well as in diabetes (reviewed in (Shantikumar, Caporali et al.) or (Battiprolu, Gillette et al.) Kumar M, Prot Cell, 2012). More unexpectedly, cardiac-specific miR-208a inhibition conferred resistance to obesity, improved glucose homeostasis and lowered plasma lipids levels in mice: an effect linked to a regulation of systemic energy homeostasis via its target MED13 (Grueter CE, Cell, 2012). In addition, other cardiovascular miRNA should be involved in the cardiac complications of diabetes.

In a previous study, the inventors demonstrated that miR-424 (ortholog to murine miR-322, which differ only by one base at the C-terminal extremity) regulated vascular smooth muscle cells (VSMC) proliferation by targeting Ca2+ regulating proteins and cyclin D1 (Merlet et al, 2013).

There is no disclosure in the art of miR-424 effects on insulin signaling in the cardiovascular system and cardiomyocytes, the protective role of miR-424 against cardiovascular consequences of hyperinsulinemia by targeting the insulin pathway, and the use of miR-424 in the prevention or treatment of diabetic cardiomyopathy.

**SUMMARY OF THE INVENTION:**

The present invention relates to a compound that raises the expression level of miR-424 for use in the prevention or treatment of diabetic cardiomyopathy in a subject in need thereof.

**DETAILED DESCRIPTION OF THE INVENTION:**

In the present invention, the inventor investigated miR-424 effects on insulin signaling in the cardiovascular system and used viral vectors to modulate its expression in the cardiomyocyte.

The inventors identified a new microRNA, human miR-424, ortholog to murine miR-322 which targets several members of the insulin signaling pathway like INSR (insulin receptor), IGFIR (IGF-1 receptor) and pro-proliferative CCND1 (cyclin D1) in vascular
smooth muscle cells. Overexpression of miR-424/322 inhibited VSMC proliferation induced by insulin. In addition AKT phosphorylation was significantly decreased as well as glucose uptake. The inventors also identified SIRT4 (sirtuin 4), shown to be implicated in fatty acid oxidation and lipid metabolism in muscles, and in insulin secretion by the pancreas as a new target of miR-424/322. Cardiac overexpression of miR-424/322 in mice using a recombinant AAV9 induced after 1 month a decrease in IGFIR, INSR, cyclin D1 proteins and a similar tendency for SIRT4 proteins. Moreover AKT phosphorylation induced by insulin was also impaired in these hearts. The inventors demonstrated that miR-424/322, by targeting the insulin pathway, may have a protective role against cardiovascular consequences of hyperinsulinemia. miR-424/322 was also overexpressed in the pancreas of the mice which showed a basal hyperinsulinemia without global insulin resistance.

Using cardiotropic adeno-associated virus constructs to overexpress or knock-down miR-322, the inventors demonstrated that miR-322 protects the heart from the consequences of hyperinsulinemia and hyperlipidemia induced by a high-fat diet. Altogether these results emphasize miR-424/322 as a new therapeutic target against diabetic cardiomyopathy, cardiac consequences of metabolic syndrome and diabetes.

**Therapeutic methods and uses**

Accordingly, the present invention relates to a compound that raises the expression level of miR-424 for use in the prevention or treatment of diabetic cardiomyopathy in a subject in need thereof.

As used herein, the term "miR-424" has its general meaning in the art and refers to the miR-424 sequence available from the data base http://microrna.sanger.ac.uk/sequences/ under the miRBase Accession number MI0001446 (pre-miR-424; SEQ ID NO: 1) and MIMAT0001341 (mature miRNA: hsa-miR-424-5p; SEQ ID NO: 2).

As used herein, the term "subject" denotes a mammal. In one embodiment of the invention, a subject according to the invention refers to any subject (preferably human) afflicted or at risk to be afflicted with diabetes. In a preferred embodiment of the invention, a subject according to the invention refers to any subject (preferably human) afflicted or at risk to be afflicted with diabetic cardiomyopathy. In one embodiment of the invention, a subject
according to the invention refers to any subject (preferably human) afflicted or at risk to be afflicted with cardiometabolic diseases.

The method of the invention may be performed for any type of diabetic cardiomyopathy, such as diabetic cardiomyopathy corresponding to abnormalities in cardiac tissue independent of vascular defects, coronary artery disease or hypertension: in particular for diabetic patients showing defects in diastolic left ventricular function, with or without systolic left ventricular dysfunction followed by left ventricular hypertrophy and fibrosis.

In one embodiment, the present invention may be performed for any type of cardiometabolic diseases. The term "cardiometabolic diseases" has its general meaning in the art and relates to cardiovascular disorders associated with metabolic syndrome, such as obesity, diabetes/insulin resistance, hypertension and dyslipidemia. The term "cardiometabolic diseases" refers to cardiac consequences of metabolic syndrome such as atherosclerosis, coronary heart disease, obesity-associated heart disease, insulin resistance-associated heart disease, hypertensive heart disease, cardiac remodeling, heart failure and cardiometabolic diseases disclosed in Hertle et al, 2014; Hua and Nair, 2014; U.S. Pat. Application No. 2012/0214771 and International Patent Application No. 2008/094939.

In a particular embodiment, the compound that raises the expression level of miR-424 may consist in an isolated, synthesized or recombinant ly miR-424.

In a particular embodiment, the compound that raises the expression level of miR-424 may consist in an isolated, synthesized or recombinant nucleic acid comprising a nucleic acid sequence that has at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with a sequence selected from the group consisting of SEQ ID NO: 1, or SEQ ID NO: 2.

Nucleic acid sequence identity is preferably determined using a suitable sequence alignment algorithm and default parameters, such as BLAST N (Karlin and Altschul, Proc. Natl Acad. Sci. USA 87(6):2264-2268 (1990)).

As used herein, an "isolated" miRNA is one which is synthesized, or altered or removed from the natural state through human intervention. For example, a miRNA naturally
present in a living animal is not "isolated". A synthetic miRNA, or a miRNA partially or completely separated from the coexisting materials of its natural state, is "isolated". An isolated miRNA can exist in substantially purified form, or can exist in a cell into which the miRNA has been delivered. Thus, a miRNA which is deliberately delivered to, or expressed in, a cell is considered an "isolated" miRNA. A miRNA produced inside a cell by a miRNA precursor molecule is also considered to be "isolated" molecule.

Isolated miRNAs can be obtained using a number of standard techniques. For example, the miRNAs can be chemically synthesized or recombinantly produced using methods known in the art. Preferably, miRNAs are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, e.g., Proligo (Hamburg, Germany), Dharmaco Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, 111, USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK) and Eurogentec (Angers, France) and Life technologies (Saint-Aubain, France).

In some embodiments, of the invention, a synthetic miRNA contains one or more design elements. These design elements include, but are not limited to: (i) a replacement group for the phosphate or hydroxyl of the nucleotide at the 5' terminus of the complementary region; (ii) one or more sugar modifications. In certain embodiments, a synthetic miRNA has a nucleotide at its 5' end of the complementary region in which the phosphate and/or hydroxyl group has been replaced with another chemical group (referred to as the "replacement design"). In some cases, the phosphate group is replaced, while in others, the hydroxyl group has been replaced. In particular embodiments, the replacement group is biotin, an amine group, a lower alkylamine group, an acetyl group, 2′0-Me (2′oxygen-methyl), DMTO (4,4′-dimethoxytrityl with oxygen), fluorescein, a thiol, or acridine, though other replacement groups are well known to those of skill in the art and can be used as well. In particular embodiments, the sugar modification is a 2′0-Me modification. In further embodiments, there is one or more sugar modifications in the first or last 2 to 4 residues of the complementary region or the first or last 4 to 6 residues of the complementary region.

In a particular embodiment, the compounds that raise the expression level of miR-424 of the invention is resistant to degradation by nucleases. One skilled in the art can readily
synthesize nucleic acids which are nuclease resistant, for example by incorporating one or more ribonucleotides that are modified at the 2'-position into the miRNAs. Suitable 2'-modified ribonucleotides include those modified at the 2'-position with fluoro, amino, alkyl, alkoxy, and O-allyl.

The present invention also relates to a vector comprising a nucleic acid sequence encoding the miR-424 according to the invention for use in the treatment of diabetic cardiomyopathy in a subject in need thereof.

Alternatively, the miRNAs can be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing RNA from a plasmid include, e.g., the U6 or HI RNA pol III promoter sequences, or the cytomegalovirus promoters. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulable promoters for expression of the miRNAs in cardiomyocytes.

The miRNAs that are expressed from recombinant plasmids can be isolated from cultured cell expression systems by standard techniques. The miRNAs which are expressed from recombinant plasmids can also be delivered to, and expressed directly in, the cardiomyocytes. The use of recombinant plasmids to deliver the miRNAs to cardiomyocytes is discussed in more detail below.

The miRNAs can be expressed from a recombinant plasmid. Preferably, the miRNAs are expressed as the miRNA precursor molecules from a plasmid, and the precursor molecules are processed into the functional miRNA by a suitable processing system, including processing systems extant within a cardiomyocyte. Other suitable processing systems include, e.g., the in vitro Drosophila cell lysate system as described in U.S. published application 2002/0086356 to Tuschl et al. and the E. coli RNAse III system described in U.S. published patent application 2004/0014113 to Yang et al, the entire disclosures of which are herein incorporated by reference.

Selection of plasmids suitable for expressing the miRNAs, methods for inserting nucleic acid sequences into the plasmid to express the gene products, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example, Zeng et al. (2002), Molecular Cell 9:1327-1333; Tuschl (2002), Nat. Biotechnol, 20:446-448; Brummelkamp et al. (2002), Science 296:550-553; Miyagishi et al. (2002), Nat. Biotechnol. 20:497-500; Paddison et al. (2002), Genes Dev. 16:948-958; Lee et al. (2002),

In one embodiment, a plasmid expressing the miRNAs comprises a sequence encoding a miR precursor RNA under the control of the CMV intermediate early promoter. As used herein, "under the control" of a promoter means that the nucleic acid sequences encoding the miRNA are located 3' of the promoter, so that the promoter can initiate transcription of the miRNA coding sequences.

The miRNAs can also be expressed from recombinant viral vectors. The RNA expressed from the recombinant viral vectors can either be isolated from cultured cell expression systems by standard techniques, or can be expressed directly in cardiomyocytes. The use of recombinant viral vectors to deliver the miRNAs to cardiomyocytes is discussed in more detail below.

The recombinant viral vectors of the invention comprise sequences encoding the miRNAs and any suitable promoter for expressing the miRNAs sequences. Suitable promoters include, for example, the U6 or HI RNA pol III promoter sequences, or the cytomegalovirus promoters or cardiac specific promoters such as cardiac Troponin T, myosin heavy chain and others. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulable promoters for expression of the miRNAs in cardiomyocytes.

Any viral vector capable of accepting the coding sequences for the miRNAs can be used; for example, vectors derived from adenovirus (AV); adenoassociated virus (AAV); lentiviruses (LV); retroviruses (Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate. For example, lentiviral vectors of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors of the invention can be made to target different cells by engineering the vectors to express different capsid protein serotypes. For example, an AAV vector expressing a serotype 2 capsid on a serotype 2 genome is called AAV 2/2. This serotype 2 capsid gene in the AAV 2/2 vector can be replaced by a serotype 5 capsid gene to produce an AAV 2/5 vector. Techniques for constructing AAV vectors which express different capsid protein serotypes are within the skill in the art; see, e.g., Rabinowitz J. E. et al. (2002), J Virol 76:791801, the entire disclosure of which is herein incorporated by reference.
Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing miR (RNA) into the vector, methods of delivering the viral vector to the cells of interest, and recovery of the expressed miR (RNA) products are within the skill in the art. See, for example, Dornburg (1995), Gene Therap. 2:301-310; Eglitis (1988), Biotechniques 6:608-614; Miller (1990), Hum. Gene Therap. 1:5-14; and Anderson (1998), Nature 392:25-30, the entire disclosures of which are herein incorporated by reference.

Preferred viral vectors are those derived from AV and AAV. A suitable AV vector for expressing the miRNAs, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia et al. (2002), Nat. Biotech. 20:1006-1010, the entire disclosure of which is herein incorporated by reference. Suitable AAV vectors for expressing the miRNAs, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski et al. (1987), J. Virol. 61:3096-3101; Fisher et al. (1996), J. Virol. 70:520-532; Samulski et al. (1989), J. Virol. 63:3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference. Preferably, the miRNAs are expressed from a recombinant AAV vector comprising the CMV intermediate early promoter.

In a particular embodiment, the miR-424 of the present invention is expressed from a recombinant viral vector targeting cardiomyocytes such as AAV9.

In one embodiment, a recombinant AAV viral vector of the invention comprises a nucleic acid sequence encoding the miR-424 precursor in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter. As used herein, "in operable connection with a polyT termination sequence" means that the nucleic acid sequences encoding the sense or antisense strands are immediately adjacent to the polyT termination signal in the 5’ direction. During transcription of the miRNA sequences from the vector, the polyT termination signals act to terminate transcription.

The compounds that raise the expression level of miR-424 can be administered to a subject by any means suitable for delivering these compounds to cardiomyocytes of the subject. For example, the compounds that raise the expression level of miR-424 can be administered by methods suitable to transfect cells of the subject with these compounds.
Preferably, the cells are transfected with a plasmid or viral vector comprising sequences encoding miR-424.

The compounds that raise the expression level of miR-424 can also be administered to a subject by any suitable enteral or parenteral administration route. Suitable enteral administration routes for the present methods include, e.g., oral, rectal, or intranasal delivery. Suitable parenteral administration routes include, e.g., intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g., intra-retinal injection, or subretinal injection); subcutaneous injection or deposition, including subcutaneous infusion (such as by osmotic pumps); direct application to the tissue of interest, for example by a catheter or other placement device (e.g., a retinal pellet or a suppository or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. Preferred administration routes are injection, infusion and direct injection into the cardiomyocytes.

In the present methods, a compound that raises the expression level of miR-424 can be administered to the subject either as naked RNA, in combination with a delivery reagent, or as a nucleic acid (e.g., a recombinant plasmid or viral vector) comprising sequences that express the miR-424. Suitable delivery reagents include, e.g, the Minis Transit TKO lipophilic reagent; lipofectin; lipofectamine; celfectin; polycations (e.g., polylysine), and liposomes.

Recombinant plasmids and viral vectors comprising sequences that express the miR-424, and techniques for delivering such plasmids and vectors to cardiomyocytes, are discussed above.

In a preferred embodiment, liposomes are used to deliver a compound that raises the expression level of miR-424 (or nucleic acids comprising sequences encoding miR-424) to a subject. Liposomes can also increase the blood half-life of the gene products or nucleic acids. Liposomes suitable for use in the invention can be formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream.

A variety of methods are known for preparing liposomes, for example, as described in Szoka et al. (1980), Ann. Rev. Biophys. Bioeng. 9:467; and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, the entire disclosures of which are herein incorporated by reference. The liposomes for use in the present methods can comprise a ligand molecule that targets the liposome to cardiomyocytes. Ligands which bind to receptors prevalent in cardiomyocytes, such as monoclonal antibodies that bind to cardiomyocyte antigens, are
preferred. The liposomes for use in the present methods can also be modified so as to avoid
clearance by the mononuclear macrophage system ("MMS") and reticuloendothelial system
("RES"). Such modified liposomes have opsonization-inhibition moieties on the surface or
incorporated into the liposome structure. In a particularly preferred embodiment, a liposome
of the invention can comprise both opsonization-inhibition moieties and a ligand.

Opsonization-inhibiting moieties for use in preparing the liposomes of the invention
are typically large hydrophilic polymers that are bound to the liposome membrane. As used
herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is
chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble
anchor into the membrane itself, or by binding directly to active groups of membrane lipids.
These opsonization-inhibiting hydrophilic polymers form a protective surface layer that
significantly decreases the uptake of the liposomes by the MMS and RES; e.g., as described
in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference.
Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-
soluble polymers with a number-average molecular weight from about 500 to about 40,000
daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers
include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy
PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-
viny pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids;
polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are
chemically linked, as well as gangliosides, such as ganglioside GM1. Copolymers of PEG,
methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the
opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino
acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The
opsonization inhibiting polymers can also be natural polysaccharides containing amino acids
or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid,
pectic acid, neuraminic acid, alginic acid, carrageenan; animated polysaccharides or
oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides,
e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups.
Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof.
Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated
liposomes".

The opsonization inhibiting moiety can be bound to the liposome membrane by any
one of numerous well known techniques. For example, an N-hydroxysuccinimide ester of
PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive animation using Na(CN)BH3 and a solvent mixture, such as tetrahydrofuran and water in a 30:12 ratio at 60° C.

Liposomes modified with opsonization-inhibition moieties remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes. Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, tissue characterized by such microvasculature defects will efficiently accumulate these liposomes; see Gabizon, et al. (1988), Proc. Natl. Acad. Sci., USA, 18:6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation of the liposomes in the liver and spleen. Thus, liposomes that are modified with opsonization-inhibition moieties are particularly suited to deliver the compounds that raise the expression level of miR-424 (or nucleic acids comprising sequences encoding miR-424) to cardiomyocytes.

The present invention also relates to the compound that raises the expression level of miR-424 according to the invention in combination with one or more anti-diabetes agent or anti-diabetic cardiomyopathy agent for use in the prevention or treatment of diabetic cardiomyopathy in a subject in need thereof.

In one embodiment, the anti-diabetes agent or anti-diabetic cardiomyopathy agent may include metformin, insulin, canagliflozin, angiotensin-converting enzyme (ACE) inhibitors, Beta blockers, Biguanide, Sulphonylureas, Alpha glucosidase inhibitor, Prandial glucose regulators, Thiazolidinediones (glitazones), Incretin mimetics, DPP-4 inhibitors (gliptins), or SGLT2 inhibitors.

The present invention also relates to a method for preventing or treating diabetic cardiomyopathy in a subject in need thereof, comprising the step of administering to said subject the compound that raises the expression level of miR-424 according to the invention.

The present invention also relates to a method for preventing or treating diabetic cardiomyopathy in a subject in need thereof comprising the step of administering to said subject a vector comprising a nucleic acid sequence encoding the miR-424.
The present invention also relates to a method for preventing or treating diabetic cardiomyopathy in a subject in need thereof, comprising the step of administering to said subject the compound that raises the expression level of miR-424 according to the invention in combination with one or more anti-diabetes agent or anti-diabetic cardiomyopathy agent.

**Pharmaceutical compositions**

The compound that raises the expression level of miR-424 of the invention may be used or prepared in a pharmaceutical composition.

In one embodiment, the invention relates to a pharmaceutical composition comprising the compound that raises the expression level of miR-424 of the invention and a pharmaceutical acceptable carrier for use in the prevention or treatment of diabetic cardiomyopathy in a subject in need thereof.

Typically, the compound that raises the expression level of miR-424 of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, inhalation, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration of the miR-424, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, inhalation administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and nasal or intranasal administration forms and rectal administration forms. Methods for preparing pharmaceutical compositions of the invention
are within the skill in the art, for example as described in Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, Pa. (1985), the entire disclosure of which is herein incorporated by reference.

The present pharmaceutical compositions comprise compounds that raise the expression level of miR-424 (e.g., 0.1 to 90% by weight), or a physiologically acceptable salt thereof, mixed with a pharmaceutically-acceptable carrier. The pharmaceutical compositions of the invention can also comprise compounds that raise the expression level of miR-424 which are encapsulated by liposomes and a pharmaceutically-acceptable carrier. Preferred pharmaceutically-acceptable carriers are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

In a particular embodiment, the pharmaceutical compositions of the invention comprise compounds that raise the expression level of miR-424 which is resistant to degradation by nucleases. One skilled in the art can readily synthesize nucleic acids which are nuclease resistant, for example by incorporating one or more ribonucleotides that are modified at the 2'-position into the miRNAs. Suitable 2'-modified ribonucleotides include those modified at the 2'-position with fluoro, amino, alkyl, alkoxy, and O-allyl.

Pharmaceutical compositions of the invention can also comprise conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include, e.g., physiologically biocompatible buffers (e.g., tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamid) or calcium chelate complexes (such as, for example, calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions of the invention can be packaged for use in liquid form, or can be lyophilized.

For solid pharmaceutical compositions of the invention, conventional nontoxic solid pharmaceutically acceptable carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For example, a solid pharmaceutical composition for oral administration can comprise any of the carriers and excipients listed above and 10-95%, preferably 25%-75%, of the compound that raises the expression level of miR-424. A pharmaceutical composition for aerosol (inhalational) administration can comprise 0.01%-20% by weight, preferably 1%-10% by weight, of the compound that raises the expression level of miR-424 encapsulated in a
liposome as described above, and a propellant. A carrier can also be included as desired; e.g., lecithin for intranasal delivery.

One skilled in the art will recognize that the amount of a compound that raises the expression level of miR-424 to be administered will be an amount that is sufficient to prevent the unwanted diseases symptoms or induce amelioration of unwanted disease symptoms. Such an amount may vary *inter alia* depending on such factors as the gender, age, weight, overall physical condition, of the subject, etc. and may be determined on a case by case basis. The amount may also vary according to the type of condition being treated, and the other components of a treatment protocol (e.g. administration of other medicaments such as steroids, etc.). If a viral-based delivery of miR-424 is chosen, suitable doses will depend on different factors such as the viral strain that is employed, the route of delivery (intramuscular, intravenous, intra-arterial, oral, inhalation or other). Those of skill in the art will recognize that such parameters are normally worked out during clinical trials. In addition, treatment of the subject is usually not a single event. Rather, the miR-424 of the invention will likely be administered on multiple occasions, that may be, depending on the results obtained, several days apart, several weeks apart, or several months apart, or even several years apart.

Pharmaceutical compositions of the invention may include any further agent which is used in the prevention or treatment of diabetes and diabetic cardiomyopathy. For example, pharmaceutical compositions of the invention can be co-administered with metformin, insulin, canagliflozin, angiotensin-converting enzyme (ACE) inhibitors, Beta blockers, Biguanide, Sulphonylureas, Alpha glucosidase inhibitor, Prandial glucose regulators, Thiazolidinediones (glitazones), Incretin mimetics, DPP-4 inhibitors (gliptins), or SGLT2 inhibitors.

In one embodiment, said additional active agents may be contained in the same composition or administrated separately.

In another embodiment, the pharmaceutical composition of the invention relates to combined preparation for simultaneous, separate or sequential use in the prevention or treatment of diabetic cardiomyopathy.

The invention also provides kits comprising the compound that raises the expression level of miR-424 of the invention. Kits containing the compound that raises the expression level of miR-424 of the invention find use in therapeutic methods.
Oligonucleotide sequences

SEQ ID NO: 1 for hsa-mir-424 MI0001446 (pre-miRNA)
5 CGAGGGGAUACAGCAGCAAUUCAUGUUUUGAAGUGUUCUAAAUGGUUCAAAA CGUGAGGCGCUCUGUACCCCCUCUCCGUGGGGAAGGUAGAAGGGG

SEQ ID NO: 2 for hsa-miR-424-5p MIMAT0001341 (mature miRNA)
CAGCAGCAUUCUCAUGUUCGAA

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: miR-424/322 is highly expressed in diabetic heart of two models of diabetes: Goto-kakizaki rats and ob/ob mice. A. Detection of miR-322 expression in various tissues of 8-months old diabetic Goto-Kakizaki (GK) rats (n=5) and 8-months old non diabetic Wistar rats (n=5). miR-322 expression was normalized to small nucleolar RNA U87. + : p<0.05, **: p<0.01.

B. Detection of miR-322 expression in heart of ob/ob mice (n=3) and control littermates (n=4). miR-322 expression was normalized to small nucleolar RNA snoR202.

Figure 2: miR-424/322 targets insulin receptors and inhibit insulin signaling in the heart.

A. Western-Blot showing mir-322 targets protein expression (insulin receptor, INSR) and IGF1 receptor (IGF1R) in the hearts of AAV infected mice (n=4/group). All proteins expression were normalized to GAPDH. *: p<0.05. B. Insulin (lui/kg) was injected in AAV infected mice. 15 min later hearts were flash frozen. After protein extraction 50µg were loaded on SDS-PAGE. Western-Blot showed AKT phosphorylation on serine 473. Quantification was determined by normalizing to total AKT in control or insulin treated mice. (n=2/group, preliminary results).
Figure 3: Consequences of miR-322 modulation on general metabolism and heart after 16 weeks of diet.

Various parameters were measured in mice after 16 weeks of diet and 10 weeks post-AAV infection with either AAVCtl, AAV322 or AAVsponge (n=9-12/group). Mean level of fibrosis measured in hearts for each group of HFD-fed mice (n=5/group) after a Sirius red staining. (HFD+AAV322 vs HFD+AAVsponge: $p<0.05$). All data are expressed as mean+/SEM.

Figure 4: miR-322 is important for cardiac function and protects the heart from HFD consequences.

3 groups of mice were fed a HFD (n=11-12/group) and 3 groups a CD (n=9-12/group) for 16 weeks. After 6 weeks of diet, AAVCtl, AAV322 or AAVsponge were injected through the tail vein. Echocardiography was performed after 6-7-10-13 and 16 weeks of diet (respectively just before AAV injection and 1-4-7-10 weeks post AAV). A. Percentage of ejection fraction (EF): HFD (dotted lines) and control diet (continuous lines). HFD-AAVctl compared to CD-AAVctl: ##: $p<0.01$, ###: $p<0.001$; CD-AAVsponge compared to CD-AAVctl: ***: $p<0.001$; HFD-AAV322 compared to HFD-AAVctl: $$$: $p<0.001$. B. Left ventricular end systolic diameter (LVESD): HFD (dotted lines) and CD (continuous lines). CD-AAVsponge compared to CD-AAVctl: **: $p<0.01$, ***: $p<0.001$; HFD-AAV322 compared to HFD-AAVctl: $: p<0.05$, $$: p<0.01$. C. Left ventricular end diastolic diameter (LVEDD): HFD (dotted lines) and CD (continuous lines). CD-AAVsponge compared to CD-AAVctl: ***: $p<0.001$. All data are expressed as mean+/SEM.

EXAMPLES:

EXAMPLE 1:

Material & Methods

Experimental animals

The Ethics Committee in Animal Experiment Charles Darwin approved this study (Ce5/2012/124). C57B16 mice, ob/ob mice as well as their control littermates and Wistar rats were from Janvier-Labs (Le Genest saint Isle, France). Mice and rats were housed in a climate-controlled environment with a 12h light/dark cycle and free access to food and water ad libitum.
Generation of adenovirus associated virus (AAV).

A miR-322 precursor DNA consisting of the mature miRNA with flanking sequences (400 bp upstream and 250 bp downstream miR-322) was PCR-amplified from rat genomic DNA using the following primers: HindIII-707bp-miR-322gene-f: AATAaagcttACCCGGGTCAATAAATGAAA (SEQ ID NO: 3) and NotI-707bp-miR-322gene-rev: GACTgcggccgcTGGGCTGAGTTCAGGATACC (SEQ ID NO: 4) and digested by HindIII and NotI. Plasmid pdsAAV2-eGFP (kind gift of Dr Xiao) was also digested by HindIII and NotI which eliminates the eGFP gene. miR-322 gene was then subcloned into pdsAAV2/HindIII-NotI under a CMV promoter. The subsequent AAV amplification was performed as previously described. Briefly 50µg pdsAAV2-miR-322 and 150µg of plasmid helper pDG-9 (allowing formation of serotype 9 AAV particles) were transfected in HEK 293T cells in presence of Lactate Ringer and polyethyleneimine (PEI). After 3 days, cells were lysed and virus collected, digested by Benzonase (Sigma-Aldrich) to eliminate free DNA, purified by ultracentrifugation on a iodixanol gradients and concentrated using Vivaspin 20 lOOKDa. The AAV-miR-322 generated were then titrated against ATCC's recombinant adeno-associated virus serotype 2 Reference Standard Material (rAAV2 RSM) using SV40 primers: f: AACCTCCCACACCTCCC (SEQ ID NO: 5) and rev: TTGGACAAACCACAACCTAGAA (SEQ ID NO: 6).

As a control a pdsAAV2-eGFP was digested by HindI which eliminates a 1250bp fragment including eGFP. After relegation this empty vector (without eGFP or transgene) was used to generate non coding AAV particles (AAV Ctl).

1.10el 1 AAV particles were injected by the tail vein and C57B16 mice were sacrificed after one month.

RNA isolation

Total RNA including miRNA were isolated with the mzVVana miRNA isolation kit (Life technologies, Villebon sur Yvette, France).

RT-PCR

miRNA specific RT-PCR were performed using specific Taqman miRNA assays (Life technologies) and normalized either to small RNA U87 for rat samples or small nucleolar snoR202 for mouse samples. The relative transcript level between two samples was calculated by using the 2^-ΔΔCT method.
Protein preparation and western blot.

Protein extracts were prepared using Promokine Mammalian Whole Cell Extraction kit (PromoCell GMBH, Heidelberg, Germany) and phosphatase inhibitors (Sigma-Aldrich). The following antibodies were used: rabbit polyclonal antibody to GAPDH (ab9485, 1/2500, Abeam, Paris, France), rabbit anti Insulin Receptor (3025, 1/1000 or 1/500, Cell Signaling), rabbit anti IGFl Receptor (3018, 1/1000, Cell Signaling), rabbit anti-Akt (4691, 1/1000, Cell Signaling) and rabbit anti phospho-Akt (Ser473) (4060, 1/1000, Cell Signaling). Densitometric analysis was performed with NIH Image/ImageJ, and the expression level of the various proteins was normalized to GAPDH ant phospho-Akt was normalized to total Akt.

Results

The inventors used prediction algorithms (miRanda: http://www.microrna.org and Targetscan 6.2: http://www.targetscan.org) to identify new target mRNA of miR-424 that could be implicated in cell metabolism and found that insulin receptor (INSR) and IGF-1 receptor (IGF1R) were potential targets.

Although miR-424/322 is considered ubiquitous, high variations in its expression were found between tissues. Interestingly miR-322 was strongly expressed in the muscle, adipose tissue and heart of rats and mice compared to aorta, liver and pancreas. Moreover cardiac miR-322 expression was specifically higher in the rodent model of type 2 diabetes (8-months old Goto-kakizaki (GK) rats) compared to same age Wistar rats whereas its expression was either unchanged or decreased in the other tissues (Figure 1A). Furthermore miR-322 was also upregulated in the heart of Ob/ob mice another well-known model of diabetes (Figure 1B).

To better understand the role of miR-424/322 in the heart and its metabolic consequences, the inventors generated a recombinant serotype 9 Adeno Associated Virus (AAV) enconding miR-322 gene or the same AAV without transgene (AAV Ctl). AAVmiR-322 infected mice displayed after one month a 10-fold increase of miR-322 expression in the heart. Cardiac miR-322 overexpression induced a decrease in INSR, IGF1R protein expression (Figure 2A). Moreover AKT-phosphorylation induced by insulin was also strongly decreased (Figure 2B).

EXAMPLE 2:

Material & Methods

Generation of adenovirus associated virus (AAV):
In addition to the methods provided in Example 1, an AAV sponge anti miR-322 was also generated. Five sites complementary to miR-322 (except for a bulge corresponding to position 9-12 of the miRNA sequence to avoid cleavage of the mRNA generated) were first subcloned by XhoI and NotI in the 3’UTR of Renilla luciferase reporter gene in psiCheck-2 vector (Promega). The oligonucleotides used were: 5XhoI-sponge322-s: ggCTCGAGTCCAAAAACATCTATGCTGCTCCGGGTCCAAAAACATCTATGCTGCTGCC GGTCCAAAAACATCTATGCTGCTGCCGGGTCCAAAAACATCTATGCTGCTGCC AAAACATCTATGCTGCGCGCCGCt (SEQ ID NO: 7) and 5NotI-sponge322-as: aaGCGGCCGCCAGCAGCATAGATGTGTGTGACCAGCGACGAGCATAGATGTTTGG ACCGGCAGC AGCATAGATGTGTGTGACCAGCGACGAGCATAGATGTTTGG ACCGGCAGCAGCATAGATGTTTGG ACCGGCAGCAGCATAGATGTTTGG ACCGGCAGCAGCATAGATGTTTGG ACCGGCAGCAGCATAGATGTTTGG ACCGGCAGCAGCATAGATGTTTGG

This construct psiCheck-2sponge allowed to check for the efficiency of the construct in vitro. Then the renilla+3’UTR miR-322sponge construct was subcloned into pdsAAV2 under the CMV promoter using Nhel and NotI restriction sites (which also eliminates eGFP) to generate AAVsponge.

In vivo experimental protocol

Rats and mice were used from Janvier-labs (Le Genest St isle, France).

The Ethics Committee in Animal Experiment Charles Darwin approved this study (Ce5/2012/124). Five-week old C57B16 mice were subjected to a normal diet (10% calories from fat, no sucrose 3.85kcal/g, D12450K) or a high-fat diet (60% calories from fat, 7% sucrose, 5.24Kcal/g, D12492) from Research Diets (Broogarden, Lynge, Denmark) for 16 weeks. Mice were housed in a climate-controlled environment with a 12h light/dark cycle and free access to food and water ad libitum. Body weight and food intake were monitored weekly.

After 6 weeks of diet, animals were anesthetized using intraperitoneal (IP) injection of ketamine (Parke Davis, France) and xylazine (Bayer, France) (75 and 10mg/kg respectively). They were injected with 1.101 l AAV particles (either AAV322, AAVsponge or AAVCtrl) through the tail vein.

Blood glucose and insulin quantification

Blood glucose levels (after a one night fasting) were determined using an Accu-Check Performa glucose analyzer (Roche, Meylan, France) with one drop of blood obtained by a tail cut. About 40µl of blood were collected in Microvette CB300 tubes containing EDTA
After centrifugation plasma samples were frozen and kept at -80°C until further analysis. Insulin concentrations were then measured using ALPCO Mouse ultrasensitive Insulin Elisa (Eurobio, Courtaboeuf, France) according to the manufacturer's instructions.

Glucose and insulin tolerance tests
For glucose tolerance tests (GTT) mice were injected IP with 2g D-Glucose (in PBS) /kg body weight after an overnight fasting. Blood glucose quantification was performed 30 minutes before and 15, 30, 60, 90 and 120 minutes after D-Glucose administration. At -30 minutes, blood samples were also collected in Microvette CB300 tubes (Sarstedt, Marnay, France) for further plasma insulin level quantification.

For insulin tolerance tests (ITT) mice were injected IP with 0.75UI human insulin (in PBS+BSA2%) (Actrapid, Novo Nordisk, La Defense, France)/kg body weight after fasting overnight. Blood glucose quantification was performed 30 minutes before and 15, 30, 60, 90 and 120 minutes after insulin administration.

Echocardiographic analysis
Echocardiographic measurements were performed on isoflurane anesthetized animals, using echocardiography-Doppler (General Electric Medical systems Co, Vivid 7 Dimension/Vivid 7 PRO) with a probe emitting ultrasounds with 9-14 MHz frequency. M-mode LV end-systolic and end-diastolic dimensions and LV percent fractional shortening and ejection fraction were averaged from at least 4-5 sets of measures obtained from different cardiac cycles.

Transcriptome analysis
Transcriptome analysis was performed on total RNA samples using the Illumina MouseWG-6 v2.0 Expression BeadChip Kit(www.illumina.com), RNA samples were processed in batches of 6 samples. 250 ng of total RNA was reverse transcribed, amplified and biotinylated using the Illumina TotalPrep RNA Amplification Kit (Ambion/Applied Biosystems). 1.5 µg of each biotinylated cRNA was hybridized to a single BeadChip at 58°C for 16-18 hours according to the manufacturer's instructions. BeadChips were scanned using the Illumina Iscan System. General data were background corrected using GenomeStudio software. Raw intensities were pre-processed in R statistical environment using the lumi package (Du et al, 2008).
Probes with detection p-values < 0.05 in at least 5% of the samples were kept for analysis. Data were first arcsinh-transformed to stabilize variance across gene expression levels (Lin et al, 2008) and then normalized according to the quantile normalization method (Du et al., 2008). The contribution and control of factors affecting gene expression was evaluated by principal component analysis. Main principal components were not associated to any particular experimental characteristic (neither microarray identifier, nor chip position).

Differential gene expression analyses were performed using the LIMMA R package (Smyth, 2004). To identify gene sets differentially associated with the tested conditions, a gene set enrichment analysis was conducted using the GeneSetDB tool (Araki et al, 2012). A hierarchical clustering, as implemented in the gplots R package, was also applied to provide illustrative support to the GeneSetDB results.

**Rat VSMC culture and treatment**

Rat VSMC were prepared using aortas from 6 week-old male wistar rats as previously described (Merlet et al, 2013). Cells were used at passages 3-8. Before insulin stimulation, rat aortic VSMC were retro-transfected either with 30 nmol/L Pre-miRTM miRNA Precursor for miR-322 (Pre-322) or Pre-miRTM Negative control (Pre-Neg) (Life Technologies) with Lipofectamine 2000 transfection reagent (Life Technologies) in 96-well plates according to the manufacturer's recommendations. After 2 days, cells were deprived of serum for 30 hours. Glucose uptake was measured using Cayman chemical Glucose uptake cell based assay (Interchim, Montlucon, France). Briefly, cells were treated with 150 nM insulin for 45 minutes and 150μg/ml 2-NBDG (fluorescent non hydrolysable analog of glucose) for 15 minutes. After washes fluorescence was measured at 535 nm. To measure proliferation, cells were cultured for 2 days in serum free-DMEM. Then, insulin (100ng/ml = 17 nM) or PBS was added for 24 hours and Bromodeoxy-Uridine (BrdU) for the last 16h. A colorimetric BrdU cell proliferation assay was performed, as recommended by the manufacturer (Roche Diagnostics, Meylan, France).

**RNA isolation and RT-PCR**

Total RNA including miRNA from cultured VSMC was isolated with the mzVVana miRNA isolation kit (Life technologies, Villebon sur Yvette, France). Alternatively total RNA from various rat tissues were prepared using the RNeasy Mini kit from Qiagen (Courtaboeuf, France) according to the manufacturer's instructions. Total RNA Reverse Transcriptase-PCR analysis was performed using the Absolute QPCR SYBR green mix
(ABgene, Courtaboeuf, France) on an MX3005P QPCR system (Stratagene, Agilent Technologies, Massy, France). Transcript levels were normalized to the RPL32 mRNA. miRNA specific RT-PCR were performed using specific Taqman miRNA assays (Life technologies) and normalized either to small RNA U87 for rat samples or small nucleolar snoR202 for mouse samples. The relative transcript level between two samples was calculated by using the 2-AACT method.

**Protein preparation and western blot.**

Protein extracts were prepared using Promokine Mammalian Whole Cell Extraction kit (PromoCell GMBH, Heidelberg, Germany) and phosphatase inhibitors (Sigma-Aldrich). The list of antibodies used is detailed in the supplemental methods. HRP-coupled secondary antibodies were used to reveal antigen-antibody complexes. Densitometric analysis was performed with NIH Image/ImageJ, and the expression level of the various proteins was normalized to GAPDH or tubulin and phospho-Akt was normalized to total Akt.

**Luciferase reporter constructs and miRNA target validation by luciferase assay.**

PsiCHECK-2 vector (Promega, Charbonnieres, France) containing both firefly and renilla luciferase genes was used to introduce 3'UTR sequence immediately downstream the stop codon of the Renilla luciferase gene. Various constructs of INSR, IGF1R and SIRT4 3'UTR were inserted with or without mutations in the region complementary to miR-424/322 seed sequence. After 48h of incubation, firefly and renilla luciferase activities were sequentially measured using the Dual-Glo Luciferase Assay system (Promega) as recommended.

**Histopathological analysis**

The heart was divided in three sections, one for RNA analysis, one for protein analysis and one for histomorphometry. The central section of the heart was fixed with 10% formalin or alternatively frozen in OCT. For fibrosis measurements, OCT-frozen hearts were sectioned into 8-μm slices, and stained with sirius red to measure fibrosis. Red coloration was detected in all heart section. Images were acquired on an Nikon Eclipse TI microscope with a DSRi1 color camera and analyzed with NIS element AR4.20.00 software using Pixelclassifier function.

**Statistical Analysis**
Data are expressed as means ± SEM. Experiments with two groups were analyzed with the non parametric Mann-Whitney test. When multiple comparisons were performed: samples were analyzed by ANOVA followed by Bonferroni post hoc test using Prism software. When p values were below 0.05, differences were considered significant.

**Results**

miR322 is highly expressed in the heart and is up-regulated in animal models of type-2 diabetes.

Although miPv-424/322 is considered ubiquitous, it showed tissue-specific variations of expression. Indeed miR-322 was strongly expressed in the heart of rats and mice as well as in muscle and adipose tissue compared to aorta, liver or pancreas. Moreover cardiac miR-322 expression was specifically higher in a rat model of type 2 diabetes (8-months old Goto-kakizaki, GK rats) compared to age-matched Wistar rats whereas its expression was either unchanged or decreased in the other tissues. It was also overexpressed in the heart of leptin deficient obese mice (ob/ob) and its expression tended to be higher in mice fed with a high fat diet (60%fat, 7%sucrose) compared to mice fed with a control diet (10%fat) for 16 weeks.

miR-322 targets several members of the insulin pathway

The inventors used prediction algorithms to identify new miR-424/322 target mRNA that could be implicated in cell metabolism and found that insulin receptor (INSR), IGF-1 receptor (IGFIR) and the downstream effector of the insulin signaling pathway MAP2K1 (MEKI) were potential targets. Sirtuin 4 (SIRT4), a protein implicated in fatty acid oxidation and insulin secretion (Haigis et al., 2006; Nasrin et al., 2010) was also a potential target.

To confirm the potency of miR-322 on these targets, miR-322 level was modulated in primary culture of rat vascular smooth muscle cells (VSMC). Over-expression of miR-322 led to a significant decrease in INSR, IGFIR, cyclin D1 (CD1) and SIRT4 protein levels whereas the level of MEKI was unchanged. On the contrary, miR-322 knock down significantly increased IGFIR, INSR, CD1, and SIRT4 protein levels when anti-miR-322 were transfected or when a sponge against miR-322 (see material and methods for details) was expressed. This effect was not correlated to mRNA regulation except for SIRT4 and CD1. To further check the direct interaction between miR-322 and its targets, various 3’UTR constructs encompassing miR-322 complementary binding sites, wild-type or mutated in miR-322 seed sequence recognition site, were tested in a reporter luciferase assay. IGFIR, INSR and SIRT4 3’UTR induced a significant decrease in Renilla luciferase expression and activity when miR-
322 was overexpressed. This inhibitory effect was lost when the 7bp seed sequence recognition site was mutated, thus indicating a direct interaction between miR-322 and these mRNA. As expected, MEK1 3'UTR did not affect Renilla luciferase activity.

The inventors also checked the effect of miR-322 over-expression on insulin-induced AKT phosphorylation. In agreement with the decrease in IGF1R and INSR expression, miR-322 over-expression also significantly blunted AKT phosphorylation. Moreover insulin-induced glucose uptake and insulin-induced VSMC proliferation were prevented.

All these results pointed towards a potential role of miR-322 as a regulator of insulin signaling and lipid metabolism. Since it was highly expressed in the heart, the inventors demonstrated that its modulation play a role during the development of diabetic cardiomyopathy.

A high fat diet induced early defects in cardiac function in mice.

First we established a model of high fat diet (HFD) induced cardiomyopathy. Mice were fed with a HFD (60% fat, 7% sucrose) or a control diet (CD) (10% fat, 0% sucrose) for 16 weeks and metabolic parameters and cardiac function were monitored. The HFD fed mice gained significantly more weight than the ones fed CD from 4 to 16 weeks and displayed a 38% overweight at the end of the protocol. Fasting glucose and insulin levels were measured at 6-9 and 14 weeks of diet. The HFD fed mice showed a trend to hyperglycemia although not significant and became progressively hyperinsulinemic. In agreement with these results, a glucose tolerance test (GTT) performed after 14 weeks showed that the HFD fed mice still regulate normally their glycemia, probably through their elevated insulin secretion. Thus after 16 weeks of diet the HFD mice seemed still in a pre-diabetic state. At the same time, cardiac characteristics were monitored by echocardiography. Ejection fraction (EF) decreased progressively and significantly after 10 weeks of HFD with a 32% decrease after 16 weeks whereas left ventricular end diastolic and systolic diameter (LVEDD and LVESD) increased progressively and significantly from 10 weeks of HFD reaching a 17% and 45% increase respectively after 16 weeks. This indicated that while in a pre-diabetic state, HFD fed mice began to show cardiac dysfunction and ventricular dilatation. In addition, at 16 weeks of diet, HFD fed mice showed a significant increase in heart weight/tibia length ratio.

miR-322 modulation using cardiotropic AAV had no impact on general metabolism.
To better understand the role of miR-424/322 in the heart and its metabolic consequences during a HFD, the inventors generated a recombinant serotype 9 Adeno Associated Virus (AAV) encoding either miR-322 gene (AAV322), the same AAV without transgene (AAVCtl) or an AAVsponge (which decrease miR-322 availability for its biological targets in the cells). After 6 weeks of diet, 1x10^11 viral particles were injected by the tail vein and mice were followed for 10 more weeks. The viral titer at the end of the protocol was similar for the three AAV in CD fed mice and slightly but not significantly lower in HFD fed mice. During the course of the diet and up to 16 weeks no difference in weight was observed between the 3 groups of AAV-injected animals. As expected, mice receiving the HFD for 16 weeks displayed a significant increase in body weight (50%) compared to CD fed mice. GTT were performed after 11 weeks of diet but despite a delayed reabsorption of glucose in HFD fed mice, no difference was observed between the 3 groups of AAV-injected animals. Similarly insulin tolerance tests (ITT) were performed after 14 weeks of diet but no significant difference was observed between all three groups of AAV-injected animals despite a decreased effect of insulin in HFD fed mice. Fasting glycemia showed a trend to be higher in HFD fed mice but did not increase during the experiment and was not different between the 3 groups of AAV-injected animals. In animals fed with HFD fasting insulinemia increased progressively whereas it did not change in mice with a CD. Animals fed with HFD receiving AAVsponge showed an early increase in fasting insulin (already significant 5 weeks post-AAV injection) which did not occur in animals injected with AAVCtl or AAV322. Finally 10 weeks post AAV and after 16 weeks of HFD, hyperinsulinemia was similar in all 3 groups of AAV-injected mice but significantly higher than in CD fed animals.

Similarly at the end of the experiment, HFD fed mice showed a significant increase in heart weight/tibia length ratio but no difference between AAV treatments. Nevertheless hearts overexpressing miR-322 showed less fibrosis than hearts where its action was decreased (Figure 3).

**miR-322 over-expression protected cardiac function during HFD whereas its inhibition was deleterious**

Cardiac function was monitored by echocardiography before AAV injection (after 6 weeks of diet defined as time 0) and 1-4-7 and 10 weeks post AAV injection. HFD induced a progressive decline in EF (already seen at time 0 compared to CD fed mice) and a progressive increase in LVESD and to a lesser extent in LVEDD in AAVCtl- and AAVsponge-injected
mice (Figure 4 A-B-C). Interestingly during the experiment AAV322-injected mice with HFD showed EF stabilization and a smaller increase in LVESD compared to AAVCtl- or AAVsponge-treated mice indicating protection from cardiac consequences of HFD. Ten weeks after AAV injection, EF in HFD fed animals treated with AAV322 was similar to EF values before AAV injection, whereas EF in HFD fed animals treated either with AAVCtl or AAVsponge were significantly lower.

In CD fed mice EF, LVESD and LVEDD from AAV322-injected animals were not different from the values observed in AAVCtl -injected animals, showing stabilized cardiac function along the course of the experiment. However mice injected with AAVsponge showed a rapid decrease in EF as well as an increase in LVESD and LVEDD (40% and 14% respectively after 10 weeks compared to values before AAV injection) reaching levels similar to HFD fed mice (Figure 4 A-B-C). These results demonstrated that the absence of miR-322 was deleterious for the normal heart.

**miR-322 modulates signaling pathways in the heart to preserve cardiac function**

At the end of the experiments, HFD mice injected with AAV322 displayed a 1.6-fold increase and HFD mice injected with AAVsponge a 1.6-fold decrease in cardiac miR-322 expression compared to HFD mice injected with AAVCTL. Previously demonstrated protein targets INSR, IGF1R, SIRT4 and CD1 were all decreased in AAV322 hearts and increased in AAVsponge hearts except for INSR.

In CD animals, AAV322 also significantly increased cardiac miR-322 expression level (2.3 fold) but only INSR and SIRT4 proteins were significantly decreased. AAVsponge mice showed a decrease in miR-322 expression level (1.6- fold) but only a trend to increase for INSR and SIRT4.

Another AAV322 over-expression experiment was performed on mice for 4 weeks and some mice were injected with insulin 10 min before sacrifice. AAV322 injection allowed a strong increase in cardiac miR-322 expression (10-fold) compared to AAVcontrol and target proteins were decreased. Interestingly, AAV322-injected mice did not respond to the burst of insulin whereas AAVCtl-injected animals did, as indicated by AKT-phosphorylation. These results suggested that insulin signaling was deregulated by miR-322 modulation in the heart.

To better understand miR-322 protective effects against cardiac dysfunction in HFD, a transcriptome analysis was performed comparing hearts of 16 weeks HFD fed mice injected either with AAV322 (which displayed preserved cardiac function), or with AAVsponge (in which the cardiac function was altered the most) for 10 weeks. The Geneset enrichment
analysis identified two pathways significantly enriched for genes with expression significantly influenced by miR-322 over-expression, the "respiratory electron transport" (False Discovery Rate (FDR) = 4.9 \(10^{-4}\)) and the "mitochondrial fatty acid beta-oxidation" (FDR = 2.1 \(10^{-3}\)) pathways. The former included 17 genes with p-values for association with miR-322 over-expression <0.05 out of the 65 genes defining the pathway. Among these 17 genes were NdufA7 (encoding NADH dehydrogenase ubiquinone 1) and Ucp3 (encoding mitochondrial proton carrier). The second enriched pathway included 13 genes among which 7 genes demonstrated statistical evidence at p <0.05 for association with miR-322 over-expression. In this gene set, Echsl encoding Emoyl coA hydratase (essential for fatty acid beta-oxidation) was the gene showing the strongest association with miR-322 over-expression. These results suggested that mitochondrial energy production, Krebs cycle and mitochondrial fatty acid beta-oxidation capacities where increased when miR-322 was overexpressed. In accordance with these results among the 22 most deregulated genes according to LIMMA analysis (Table 3) Ucp3, Echsl and Ndufa7 were found. Pari (Presenilin associated, rhomboid like), known to be decreased in diabetes was also upregulated. Interestingly the most significantly downregulated gene was Pea15, known to be overexpressed in type 2 diabetes and to decrease insulin action.

These results demonstrated that miR-322 was also indirectly involved in improvement of mitochondrial capacities in fatty acid metabolism.

**Table 3: most significantly deregulated genes in the heart of HFD-AAV322 and HFD-AAVsponge mice.**

Limma analysis was performed on the results of transcriptome data to compare expression in the heart of HFD-AAV322 mice (showing cardioprotection) and HFD-AAVsponge mice (with major cardiac dysfunction) (n=3/group). The 22 most significant genes deregulated are presented (HFD-AAV322/ HFD-AAVsponge). Genes in bold are of particular interest regarding mitochondrial function and fatty-acid metabolism.

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REFERENCES:
Throughout this application, various references describe the state of the art to which this invention pertains.


CLAIMS:

1. A method for preventing or treating diabetic cardiomyopathy in a subject in need thereof comprising the step of administering to said subject a compound that raises the expression level of miR-424.

2. The method according to claim 1 comprising the step of administering to said subject a vector comprising a nucleic acid sequence encoding the miR-424.

3. A pharmaceutical composition comprising the compound according to claim 1 or the vector according to claim 2 for use in the prevention or treatment of diabetic cardiomyopathy in a subject in need thereof.
Figure 1
Figure 2
Figure 3
Figure 4
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV.** A61K48/00 C12N15/864

**ADD.**

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>wo 2010/097471 A2 (NAT UNIV IRELAND [I E]; SAMALI AFSHIN [I E]; GUPTA SANJEEV [I E]) 2 September 2010 (2010-09-02) paragraphs [0018], [0038]; claims 11, 13, 14, 16, 18, 19</td>
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 Further documents are listed in the continuation of Box C.  

**See patent family annex.**

**Date of the actual completion of the international search**

30 March 2015

**Date of mailing of the international search report**

13/04/2015

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**Authorized officer**

Chambonnet, F

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