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(54) Title: PANEL OF MICRORNA BIOMARKERS IN HEALTHY AGING

(57) Abstract: Methods are provided for determining if a subject is likely to develop an age-related disease based on miRNA signatures. Related methods of treatment are also provided.

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PANEL OF microRNA BIOMARKERS IN HEALTHY AGING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 61/791,426, filed March 15, 2013, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Throughout this application various publications are referred to, including by number in parentheses. Full citations for these references may be found at the end of the specification. The disclosures of these publications, and of all patents, patent application publications and books referred to herein, are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[0003] Since the introduction of high throughput technology to measure genome-wide gene expression levels, mounting evidence in model organisms indicates that aging is accompanied by enhanced gene expression variation (26,27) and a decline in gene co-expression network integrity (28,29). These results suggest that aging may affect major gene expression regulators leading to deregulation of many downstream targets, having a major impact on cell and tissue function, disease risk, and lifespan. Recently, miRNAs have emerged as critical regulators of gene expression and have been linked to longevity (30,31) and aging (32) in *C. elegans*.

[0004] MicroRNAs (miRNAs), first discovered in *C. elegans* (33), are small non-coding RNA species that post-transcriptionally regulate gene expression (34). Mature miRNAs, 18-25 bp in length, are transcribed as primary-miRNA (pri-miRNA) molecules containing a characteristic stem loop structure. This stem loop targets pri-miRNA for processing by a number of RNAses, namely Drosha and Dicer, which produce a short RNA duplex (34). From the duplex, one or both strands are incorporated into the RNA inducing silencing complex (RISC), resulting in an active miRNA. The active miRNA primarily target the 3' UTR of a mRNA based on sequence homology (35). The nucleotides in the 2-7 position of the 5' end of the mature miRNA comprise a "seed region." Absolute homology in this region is required for miRNA to target a given mRNA (36). Once an mRNA is targeted by a

miRNA, its gene expression is down-regulated due to induction of mRNA degradation or by blocking translation through conserved mechanisms (34,37). Since one miRNA can bind multiple mRNA targets, miRNAs can significantly alter gene regulatory networks. In-depth study and characterization of miRNA impact has elucidated their critical functions in development, homeostasis, and disorders including cardiovascular (38) and neurodegenerative disease (39). Thus far, 1048 human miRNA sequences have been identified through cloning, sequencing, or computational analysis (mirBase, release 16, 2010) (40,41) and *in silico* analysis predicts that they may regulate up to 1/3rd of the human genome (42).

[0005] Multiple miRNAs have been shown to regulate life span of *C. elegans* both positively and negatively (30,31,43) adding weight to the hypothesis that this gene class may contribute to robustness required for maintenance of healthy life span (44). For example, reducing the activity of miRNA, *lin-4*, shortened life span and accelerated tissue aging, whereas overexpression of *lin-4* extended life span by suppressing the target gene, *lin-14* (30). Furthermore, expression patterns of these lifespan-modulating miRNAs can be a predictor of lifespan in *C. elegans* (43); they control gene expression involved in major conserved pathways that impact life span, such as the insulin/IGF-1 signaling pathway (30,31,43). Recently, miRNAs were shown to mediate the longevity phenotype in mammals, namely, Ames dwarf mice (45), implicating a role in mammalian longevity. Since a significant number of miRNAs are evolutionarily conserved (46,47), regulation of longevity by miRNAs is expected in humans. Indeed, several human miRNAs target components of well-known conserved longevity pathways (32) including IGF (miR-1, miR-7, miR-122, miR-206 miR-320, and miR-375) (48,49,50) steroid (miR-122, miR-14, let-7) (32,51,52) and target of rapamycin (TOR) (miR-21) (53) signaling (Fig. 1). In addition, some of these miRNAs have been linked to human aging-related disorders such as heart (54-64), muscle (59), and neurodegenerative disease (65,66) (Fig. 1).

[0006] The present invention addresses the need for elucidating the role of miRNAs and their target genes in human longevity, and their impact on age-related diseases.

SUMMARY OF THE INVENTION

[0007] A method is provided for determining if a subject is likely to develop an age-related disease comprising determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21,

miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not likely to develop an age-related disease when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA.

[0008] Also provided is a method for treating a subject for an age-related disease comprising determining if a subject is likely to develop an age-related disease comprising a) empirically determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not suitable for treatment when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA, and as suitable for treatment when the sample contains levels of the miRNAs below the respective predetermined control levels for each mRNA, and b) administering to a subject who has been identified as suitable for treatment in a) a treatment for an age-related disease, so as to thereby treat the subject.

[0009] Also provided is a method for treating a subject for an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to treat an age-related disease in a subject.

[0010] Also provided is a method for reducing the risk that a subject will suffer an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to reduce the risk that a subject will suffer an age-related disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Fig. 1: MiRNAs involved in conserved pathways of longevity and their role in age-related diseases in humans.

[0012] Fig. 2. Steps involved in miRNA discovery using massively parallel sequencing and development of an automated analytical pipeline.

[0013] Fig. 3. Expression of miRNAs in (A) LCLs (Lymphoblastoid cell lines) that are significantly different between centenarians (n=20, mean age 101 yrs) and controls (n=20, mean age 74 yrs) with Fold Change >1.5 and FDR <0.05, and (2) plasma that are

significantly different between centenarians (n=10, mean age 98.5 yrs) and controls (n=10, mean age 74.7 yrs) with Fold Change >5.0 and FDR <0.05.

[0014] Fig. 4A-C. Validation of longevity-associated miRNAs. Cross sectional analysis of miRNA expression patterns at different ages can differentiate whether a miRNA is A) age-related, B) longevity-associated with youthful preservation, C) Cross sectional expression patterns of hsa-miR-29c suggest the youthful preservation model.

[0015] Fig. 5. Average relative expression of miR-20a over 3 independent measurements by TaqMan qPCR in 2 centenarian LCLs. The lines are SD. CVs (mean/SD of 3 measurements) are indicated.

[0016] Fig. 6. IGF1 pathway subnetwork of longevity-associated miRNAs (red dots). Lines link miRNAs and their target (blue dots).

[0017] Fig. 7. IGF1R 3' UTR targeted by multiple miRNAs.

[0018] Fig. 8A-B. Down-regulation of genes involved in IGF1 signaling (A) and significant reverse-correlations between these genes and longevity-associated miRNAs (B); centenarians: Red dots, controls: Blue dots

[0019] Fig. 9A-C. Network analyses. (A) Embedment of a group of functionally related genes in a base biological network. (B) Construction of the subnetwork as defined by the embedded genes and the underlying base network. Additional related genes are identified. (C) Identification of modules within the subnetwork. Modules are shown as groups of encircled green nodes.

[0020] Fig. 10. Luciferase 3'UTR reporter assays to determine molecular interactions between a miRNA and its target genes.

[0021] Fig. 11A-C. Downregulation of IGF1 gene expression (A) and AKT phosphorylation (B) in LCLs of centenarians harboring longevity-associated miRNA signature as compared to LCLs from centenarians without the signature. Reverse-correlation (C) of all individuals; centenarians (Red) and controls (Blue).

[0022] Fig. 12A-E. Effects of miR-142 overexppression on IIS and mTOR signaling in MCF7 cells. (A) Reduced IIS as measured by phosphorylation of IGF1R, AKT, and FOXO3 in response to IGF1 treatment. (B) Quantification of (A). (C) Reduced protein levels of INSR, IGF1R, and RICTOR. (D) Quantification of (C). (E) Reduced mRNA expression of INSR, PI3KR2, RICTOR, and mTOR by qPCR.

[0023] Fig. 13A-C. RICTOR is a direct target of miR-142. (A) No. of *in silico* predicted miR-142 targets. (B) 3'UTR reporter assays of RICTOR 3'UTR fragments. (C) Pull-down assay of Bi-miR-142.

DETAILED DESCRIPTION OF THE INVENTION

[0024] A method is provided for determining if a subject is likely to develop an age-related disease comprising determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not likely to develop an age-related disease when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA.

[0025] Determining, as used herein, means experimentally determining, for example, using a machine or device, testing empirically.

[0026] Also provided is a method for treating a subject for an age-related disease comprising determining if a subject is likely to develop an age-related disease comprising a) empirically determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not suitable for treatment when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA, and as suitable for treatment when the sample contains levels of the miRNAs below the respective predetermined control levels for each mRNA, and b) administering to a subject who has been identified as suitable for treatment in a) a treatment for an age-related disease, so as to thereby treat the subject.

[0027] In an embodiment of the methods, when the sample contains levels of the miRNAs below the predetermined control levels for each mRNA, the subject is identified as likely to develop an age-related disease.

[0028] In an embodiment of the methods, the sample comprises plasma or cell-free serum. In an embodiment of the methods, the sample comprises lymphoblastoid cells.

[0029] In an embodiment of the methods, a subject is identified as not likely to develop an age-related disease when all of miR-142, miR-101, miR-301b, miR148a, miR21, miR-

29c, miR30e, miR27a and miR15a are at a level above their respective predetermined control levels.

[0030] In an embodiment of the methods, a subject is identified as likely to develop an age-related disease when all of miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a are at a level below their respective predetermined control levels.

[0031] In an embodiment of the methods, the method further comprises testing a sample from a subject identified as likely to develop an age-related disease with a test predictive of development of, or predisposition to type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension, cognitive impairment, obesity, atherosclerosis, muscle atrophy or a neurodegenerative disease.

[0032] In an embodiment of the methods, the method further comprises treating a subject identified as likely to develop an age-related disease with a prophylactic treatment for an age-related disease.

[0033] In an embodiment of the methods, the method further comprises treating a subject identified as predisposed to, or likely to type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension or cognitive impairment with a treatment for type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension, cognitive impairment, obesity, atherosclerosis, muscle atrophy or a neurodegenerative disease, respectively.

[0034] In an embodiment of the methods, the age-related disease is type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension or cognitive impairment. In an embodiment of the methods, the age-related disease is cardiovascular disease and is stroke, myocardial infarction, or a coronary vascular disease.

[0035] Hypertensive subjects, in an embodiment, are considered as those with self-reported pharmacological treatment or those who meet the criteria of The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, specifically, systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg. Type 2 diabetes mellitus (T2DM) in an embodiment is subjects on pharmacological treatment or using American Diabetes Association criteria of fasting glucose ≥ 126 mg/dl, and HbA1C> 6.5%. Subjects with cardiovascular diseases, in an embodiment, are subjects with a history of acute non-fatal myocardial infarction, stroke and cardiac surgeries including angioplasty or coronary bypass surgery. Metabolic Syndrome

subjects, in an embodiment, are subjects defined using the criteria of the National Cholesterol Education Program modified Adult Treatment Panel III Report, namely the presence of three or more of the following five attributes: waist circumference exceeding 102 cm (men) or 88 cm (women), triglycerides levels > 150 mg/dl, HDL cholesterol < 40 (men) or 50 (women), blood pressure ≥130/85, history of diabetes or glucose >100 mg/dl. Cognitive impairment (MCI/dementia) and test scores on neuropsychological tests are based on the Clinical Core procedures used in the Einstein Aging Study and overlaps substantially with the Uniform Data Set of the Alzheimer's Disease Centers. These neuropsychological tests are standardized, well-normed and divided into partially overlapping domains to establish clinical diagnoses.

[0036] Also provided is a method for treating a subject for an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to treat an age-related disease in a subject. Also provided is a method for reducing the risk that a subject will suffer an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to reduce the risk that a subject will suffer an age-related disease.

[0037] In an embodiment, the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a is administered systemically. In an embodiment, the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a is administered intravenously. In an embodiment, the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a is administered in a pharmaceutically acceptable carrier. In an embodiment, the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a administered is a locked nucleic acid miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a. A locked nucleic acid is a high-affinity RNA analog in which one or more of the ribose rings are "locked" in the ideal conformation for Watson-Crick binding. As a result, locked nucleic acid microRNAs exhibit high thermal stability when hybridized to a complementary DNA or RNA strand and also exhibit high stability in serum. In embodiments, the locked nucleic acid microRNA contains one, two or three modified ribose rings. Specifically, the ribose ring is connected by a methylene bridge between the 2'-O and 4'-C atoms. In an embodiment, the locked nucleic acid microRNA is administered with

the sequence of a microRNA precursor. In an embodiment, the locked nucleic acid microRNA is administered with the sequence of a mature microRNA.

[0038] In an embodiment, the miR-142 is administered. In an embodiment, the amount of miR-142 administered is sufficient to decrease IGF1 signaling in a subject.

[0039] In an embodiment, the microRNA administered has the same sequence as a corresponding human microRNA. For example, the miR142 administered has the same sequence as a human miR142.

[0040] In an embodiment, the age-related disease is type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension or cognitive impairment. In an embodiment, the age-related disease is cardiovascular disease and is stroke, myocardial infarction, or a coronary vascular disease.

[0041] In an embodiment of the methods described herein, the subject is a mammal. In a preferred embodiment, the subject is a human subject.

[0042] The methods described herein are useful in various settings. For example, in determining death risk of an individual in the case of a life insurance policy application, a determination that the individual shows the 9 miRNA signature at levels higher than control would suggest a good risk situation for the insurance carrier company.

[0043] In an embodiment, the miRNAs are of the miRNA precursor sequences as set forth in the Experimental Results section below. In an embodiment, the miRNA has a sequence as set forth in one of SEQ ID NOS:1-9. In an embodiment, the miRNA has a sequence as set forth in one of SEQ ID NO:1 or a mature form thereof.

[0044] As used herein, a predetermined control level is a value decided for a control system or entity. The concept of a control is well-established in the field, and can be determined, in a non-limiting example, empirically from non-afflicted subject(s) (versus afflicted subject(s)), such as an age-appropriate healthy subject. The predetermined control level and may be normalized as desired to negate the effect of one or more variables.

[0045] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0046] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

[0047] The multitude of important roles played by miRNAs indicates that they are a critical genetic component of gene regulatory networks. However, quantification of miRNA has been technically challenging due to their small size, low copy number, interference from other small RNAs, and contamination by degradation products of mRNAs or other RNA species. Until recently, the only known and computationally predicted miRNAs have been interrogated using hybridization-based array methods, an assay of limited value due to cross-hybridization, array content, and the inability to discover novel miRNAs. Increased availability and affordability of massively parallel sequencing offer a dramatically improved method to gain a high-resolution view of miRNA expression (67). This technology has been utilized to quantify expression profiles of miRNAs in several species, including humans (68,69).

[0048] The discovery of miRNAs points to an entirely new regulatory module for control of biological processes. Increasingly, studies are linking altered miRNA function to disease mechanisms (70). It is hypothesized herein that miRNAs play a major role in modulating human lifespan and the aging process. This has been the case in some studies of model organisms (30,31,43). The important roles for miRNAs in human longevity disclosed herein provide a rational basis for intervention strategies using miRNA therapeutics that promote healthy aging. This is based on the fact that in contrast to other cellular mediators, miRNAs can be easily manipulated and therapies based on anti-miRs or miRNA mimics developed to repress pathological miRNAs (71,72) or overexpress protective miRNAs (38).

[0049] An innovative study design was effected involving a unique cohort of centenarians, their offspring, and age-matched, sex-matched controls without a family history of exceptional longevity, all of genetically homogeneous Ashkenazi Jewish (AJ) descent, collected by Dr. Nir Barzilai of Albert Einstein College of Medicine of Yeshiva University. The concept of miRNA regulation as a factor involved in extreme human longevity is novel. The determinations disclosed herein that there is a difference in miRNA expression levels in LCLs and plasma from centenarians as compared to controls opens up a new approach for studying modulation of longevity in humans. Also, the approach used herein for investigating the role of miRNAs in human longevity is novel. This combines an unbiased genome-wide discovery approach utilizing cutting-edge technologies for discovery of longevity-associated miRNAs and association/mechanistic studies using advanced methods to ascertain their functional relevance and biological significance.

[0050] The hypothesis that the maintenance of youthful miRNA expression patterns is beneficial and long-lived humans (e.g. centenarians) are enriched with "longevity-promoting" miRNAs that confer robustness to gene expression regulatory networks protecting against age-related deterioration was tested. Longevity in humans is an inherited trait. While the heritability of average life expectancy has been estimated to be only ~25% (79,80), studies of centenarians indicate much stronger heritability at old age. For example, siblings of centenarians have a 4 times greater probability of surviving to age 90 than siblings of those with average life span (81). Living to age 100 is 17 and 8 times more likely for male or female siblings of centenarians, respectively, compared to their birth cohort (82). In addition, longevity is strongly inherited from parents whose age of death is over 70, and more so as age of parents' death increases, but not with parents who die before age 70 (81). These findings firmly established the utility of human centenarians as a model system to study the genetics of aging and longevity. Thus, genetic studies of centenarians are based on the premise that such research may help identify genetic factors that are either particularly enriched in these populations, due to positive effects on life span, or under-represented due to a negative impact on health. Indeed, centenarians show "positive phenotypes of aging", including extended preservation of function, such as cognitive and vascular function, and resistance to age- related disease and frailty (73-78). Since the frequency of centenarians is only ~1/10,000 individuals, the longevity factors may not be present in a younger (~60-70 yrs) control population without a family history of longevity.

[0051] Study Population: AJ centenarians, their offspring, and controls. The genetically homogenous populations of Ashkenazi Jews (AJ) were studied and biological samples and phenotype data was collected from centenarians, their offspring and unrelated controls. The rationale of this study design is that if longevity is inherited, longevity-associated, measurable clinical and biological phenotypes can also be identified in the offspring of centenarians at an early age. Indeed, plasma high-density lipoprotein (HDL) cholesterol levels and lipoprotein particle sizes are dramatically higher in the offspring of centenarians (83,84) and are correlated with the cognitive function of centenarians (85). Several studies demonstrated that the offspring of centenarians have a markedly reduced prevalence of age-related diseases, such as cardiovascular disease, diabetes mellitus, and cancer, as compared to unrelated age-matched controls (76,86,87). These studies suggest that survival to exceptional old age may involve lower susceptibility to a broad range of age-related diseases, perhaps secondary to inhibition of basic mechanisms of aging. Thus, centenarian-

enriched genotypes and molecular phenotypes such as gene expression levels in the offspring of centenarians suggest that this population can be used to test the heritability of exceptional longevity using age-matched controls. The genetic homogeneity of the AJ population contributes to the enhanced likelihood of successfully identifying genetic components of aging and longevity (88). The study population derives from The Longevity Genes Project (LGP). The subjects were already phenotyped with stored DNA and LCLs of AJ proband centenarians (n=542, >95), their offspring (offspring of parents with exceptional longevity, n=691, ages 60-85), and age- and gender-matched controls (offspring of parents with usual survival, n=601, ages 60-95).

[0052] LCLs for gene expression analysis. In this study, miRNAs with "general", rather than tissue-specific, patterns of gene expression associated with human longevity were discovered as they are likely to be involved in "common" aging pathways (19). LCLs established from LGP subjects were studied because recent studies, including in this laboratory (1,2) have demonstrated that LCLs reflect functional characteristics of the donor and can be a useful tool for studying genotype-driven molecular endpoints such as gene expression, and expression quantitative trait locus (eQTL) analysis (3,4). Use of LCLs is justified because: 1) gene expression studies in various cell types, including LCLs, demonstrated that a large fraction of gene expression patterns are shared across different cell types (5); 2) LCLs act as surrogate tissues whenever there is correlation between the expression levels of LCLs and phenotypes of interest (6,7); 3) LCLs are an effective tool to identify disease genes by genome-wide eQTL analysis (8-15); and 4) there is increasing evidence that a large number of eQTLs originally identified in LCLs can also be detected in multiple primary tissues (16-18). Thus, studies in LCLs have been helpful for identifying functional regulatory variation and will be integral to improving understanding of genetics of gene expression in humans. Only positive results are interpreted, as in most large-scale discovery-based science (such as association studies). Expression profiling in LCLs provides a cost-efficient approach for identification of novel longevity-associated miRNAs, without the substantial cost, risk or inconvenience of collecting tissue from subjects (a logistically difficult task, unlikely to achieve adequate participation).

[0053] Plasma for miRNA analysis. Recent studies have revealed that miRNAs circulate in a cell-free form in blood (89,90) where they are relatively stable due to binding with other materials such as exosomes (91,90). Moreover, tissue miRNAs are released into circulating blood, serum or plasma. Such cell-free miRNAs can be studied as biomarkers

for diverse diseases including cancers and cardiovascular disease (54, 90-97). MiRNA signatures in blood are similar in men and women (89), miRNA levels are similar in plasma and serum (91), and freeze/thaw as well as prolonged storage do not affect miRNA levels (91).

Experimental Results

Example 1

[0054] 1) Discovery of miRNAs that are differentially expressed in LCLs of centenarians vs. controls. Preliminary work resulted in miRNA-seq and differential expression analysis of 3 centenarians (mean age 104) vs. 3 younger controls (mean age 63 controls). This was expanded to discover all possible miRNAs differentially expressed between 20 centenarians (mean age 101) and 20 controls (mean age 74 controls). 12-multiplex miRNA-seq was performed of individually barcoded libraries by Illumina Hi-Seq2000, which yielded a total of 2.7 x 10^8 reads from centenarians and 3.1 x 10^8 reads from controls. After removal of low quality reads and redundancy, there was a total of 1.1 x10^6 and 1.0 x10^6 unique reads for the centenarians and the controls, respectively. To analyze the computationally challenging miRNA-seq data, an automated analytical pipeline was developed (Fig. 2). Briefly, the sequencing data was provided from the Hi-Seq2000 sequencer in a standard fastq forma (98). Fastq files were trimmed of adapter sequences and low quality reads (more than 3 low quality base-calls), through a C++ program. These sequences were then collapsed to remove redundancy using the Galaxy Genome Browser tool fastx (99), followed by alignment to the known human miRNA/small RNA database or were put into the mirDeep pipeline for the discovery of novel miRNA (100). The miRNA tags matched were statistically normalized on a tags (determined miRNA) per total read (result from sequencer) basis (67). Following normalization, stringent criteria were applied for a miRNA to be considered for linear analysis, namely to be present in at least 50% (n=20) of the samples in greater than 10 copy numbers. After square-root transformation of data, a t-test was performed to generate nominal p-values (101). Correcting for multiple testing by a permutation procedure (102,103), miRNAs with a false discovery rate (FDR) <0.05 were considered significantly differentially expressed. A total of 37 miRNAs met this cutoff with a fold change >1.5, 28 of which had a fold change > 2.0. Average read numbers for the 37 significant miRNAs ranged from 10 to over 480,000 (Table A1) with up to a 46-fold change. Of these 37 miRNAs, 26 have increased expression in centenarians. Fig. 4A is

a heat map showing relative expression of the 37 significant miRNAs in controls and centenarians.

[0055] 2) Cross platform comparison of differential miRNA expression. qRT-PCR analysis was conducted using TaqMan probes (Applied BioSystems) to compare the expression of differentially expressed miRNAs detected by Illumina sequencing (Appendix 1 and date not shown). While TagMan qPCR validated sequencing results, it clearly was less sensitive and specific than miRNA-seq in detecting relative expression and fold change. Nevertheless, qPCR method can reproducibly detect differential expression when read numbers of a miRNA are >100 and fold change >2.0.

[0056] 3) Cross sectional analysis of miRNA expression in different age groups. Since the preliminary results were generated by differential analysis of two age groups, the mode of differential expression was determined. If up-regulation is simply age-related, expression will increase monotonically with age (Fig. 4A). In contrast, if up-regulation is longevity-related, patterns of youthful expression will be preserved (Fig. 4B). Such miRNA was found by a cross-sectional analysis in LCLs using TaqMan qPCR; the expression levels of miR-29c significantly decline with age (from 70s to early 90s) in control individuals while centenarians maintained the "highest" expression levels, suggesting that miR-29c is a longevity-associated miRNA (Fig. 4C).

[0057] 4) Discovery of longevity-associated miRNA in plasma by TaqMan miRNA arrays. Recent studies have shown that circulating miRNAs can be profiled as biomarkers from small amounts of total RNA of serum or plasma using TaqMan qRT-PCR arrays (104-106). The TaqMan Human MicroRNA Array Panel A+B (Applied Biosystems), which detects 664 mature miRNAs and miRNAs* present in the Sanger mirBase, was used to profile miRNA expression in plasma samples of 10 centenarians (mean age 98.5) and 10 controls (mean age 74.7, controls). To isolate total RNA from plasma, the protocol by Mitchell et al. (91) was used with a slight modification using the mirVana PARIS kit (Roche). 200 µl of plasma as starting material, which provides a yield of >0.2 µg of small RNAs was used. Pre-amplification using the TaqMan PreAmp Mater Mix (Applied Biosystems) was performed to generate a miRNA cDNA library from each plasma sample, from which miRNA profiling was carried out (ABIPrism 7900HT). Data were analyzed with SDS Relative Quantification Software (v 2.3, Applied BioSystems). Mammalian U6 embedded in TaqMan Human MicroRNA Arrays was used as an endogenous control to normalize expression signaling. Relative expression levels of miRNAs were calculated

using the comparative ΔΔCt method (107,108) followed by log2-transformation. In order for a miRNA to be considered for differential analysis, it was required to be detected in at least 8 of the 20 samples. Fold changes in miRNAs were calculated by the equation 2-ΔΔCt. Statistical significance was determined using the Mann-Whitney test with multiple testing corrections by Benjamini-Hochberg method (109) to control for false discovery rate (FDR). MiRNAs with FDR < 0.05 were considered significant. A total of 65 differentially expressed miRNAs with fold change >2.0 were discovered, among which 49 miRNAs show fold change >5.0 (Fig. 2B and Table 2). Interestingly, all these miRNAs have increased expression in centenarians.

[0058] MiRNA precursor sequences are set forth below:

hsa-mir-142 MI0000458

GACAGUGCAGUCACCCAUAAAGUAGAAAGCACUACUAACAGCACUGGAGGGU GUAGUGUUUCCUACUUUAUGGAUGAGUGUACUGUG

(SEQ ID NO:1)

hsa-mir-101-1 MI0000103

UGCCCUGGCUCAGUUAUCACAGUGCUGAUGCUGUCUAUUCUAAAGGUACAGU ACUGUGAUAACUGAAGGAUGGCA

(SEQ ID NO:2)

hsa-mir-301b MI0005568

GCCGCAGGUGCUCUGACGAGGUUGCACUACUGUGCUCUGAGAAGCAGUGCAA UGAUAUUGUCAAAGCAUCUGGGACCA

(SEQ ID NO:3)

hsa-mir-148a MI0000253

GAGGCAAAGUUCUGAGACACUCCGACUCUGAGUAUGAUAGAAGUCAGUGCAC UACAGAACUUUGUCUC

(SEQ ID NO:4)

hsa-mir-21 MI0000077

UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACC AGUCGAUGGGCUGUCUGACA

(SEQ ID NO:5)

hsa-mir-29c MI0000735

AUCUCUUACACAGGCUGACCGAUUUCUCCUGGUGUUCAGAGUCUGUUUUUGU CUAGCACCAUUUGAAAUCGGUUAUGAUGUAGGGGGA

(SEQ ID NO:6)

hsa-mir-30e MI0000749

GGGCAGUCUUUGCUACUGUAAACAUCCUUGACUGGAAGCUGUAAGGUGUUCA GAGGAGCUUUCAGUCGGAUGUUUACAGCGGCAGGCUGCCA

(SEQ ID NO:7)

hsa-mir-27a MI0000085

CUGAGGAGCAGGGCUUAGCUGCUUGUGAGCAGGGUCCACACCAAGUCGUGUU CACAGUGGCUAAGUUCCGCCCCCCAG

(SEQ ID NO:8)

hsa-mir-15a MI0000069

CCUUGGAGUAAAGUAGCAGCACAUAAUGGUUUGUGGAUUUUGAAAAGGUGC AGGCCAUAUUGUGCUGCCUCAAAAAUACAAGG

(SEQ ID NO:9)

Materials and methods.

[0059] 1) Discovery of longevity-associated miRNA in LCLs by miRNA-seq and in plasma by TaqMan miRNA arrays. To discover miRNAs associated with longevity in humans, miRNA-seq by Illumina Hi-Seq2000 is employed to comprehensively analyze all possible miRNAs expressed in LCLs, and TaqMan miRNA arrays for plasma miRNAs. 80 individuals are selected from controls at different ages uniformly distributed from 60-90 and 20 centenarians (total 100) for discovery. The sample size gives reasonable statistical power to account for individual variation in expression levels (Table 1).

[0060] Table 1: Statistical power of monotonicity test.

Stage	Sig. Level	Difference between 90 yr controls and centenarians	Power
Discovery (n=100)	0.05	0.75 SD	0.80

[0061] 2) Validation of longevity-associated miRNAs. The longevity-associated miRNAs are validated based on cross-sectional expression patterns. Since preliminary results indicated that significantly differentially expressed miRNAs are mostly upregulated in centenarians as compared to controls (Fig. 4), upregulation is used as a model. If up-regulation is simply age-related, expression will increase monotonically with age in all individuals (Fig. 4A). In contrast, if up-regulation is longevity-related, patterns of youthful

expression will be preserved both in centenarians and offspring (Fig. 4B). Also considered is the presence of significantly down-regulated miRNAs in centenarians with youthful maintenance of expression patterns, namely increased expression with age in controls but low levels of expression in centenarians and offspring. TaqMan qPCR analysis of longevity-associated miRNAs discovered in LCLs is conducted using LCL samples from 500 centenarians, 500 offspring, and 500 controls at various ages. Similarly, TaqMan qPCR analysis of longevity-associated miRNAs discovered in plasma using plasma samples from 500 centenarians, 500 offspring, and 500 controls at various ages. As described previously, for plasma miRNAs TaqMan PreAmp Master Mix and miRNA assay kit is used with spiked-in synthetic *C. elegans* miRNAs a signal normalizer. Two-tailed two sample Student's t tests and ANOVA are used for statistical evaluation. The top 20 longevity-associated miRNAs discovered in LCLs and plasma are used for validation analysis, prioritized based on fold change, read numbers, biological relevance to aging and longevity according to their predicted and validated target genes as well as overlap between the LCLs and plasma results. The results based on comparison between centenarians and controls (age, 70s) indicate that a total of 9 miRNAs were up-regulated both in LCLs and plasma of centenarians compared to controls (Table 2), including the candidate longevity-associated miRNAs, miR-29c (Fig. 5C), and miR-101, miR-148a, and miR-27a, all of which were shown to be down-regulated with age in PBMCs (110).

[0062] Table 2. MiRNAs up-regulated both in LCLs and Plasma of centenarians as compared to controls with fold change. FDR <0.05

miRNAs	Fold Change	
	LCLs	Plasma
hsa-miR-142	18.86	10.84
hsa-miR-101	9.23	5.83
hsa-miR-301b	5.94	5.06
hsa-miR-148a	3.6	5.51
hsa-miR-21	3.45	5.05
hsa-miR-29c	2.58	5.64
hsa-miR-30e	2.27	6.63
hsa-miR-27a	2.06	5.95
hsa-miR-15a	1.82	28.45

[0063] Data Analysis and Statistical Consideration. To analyze LCL miRNA-seq data, an automated analytical pipeline is used (Fig. 2). Expression profiling of each subject is normalized by its total number of reads. Square root transformation is applied to the normalized read count for regression analysis described below. To analyze plasma TaqMan miRNA array Data, SDS Relative Quantification Software (v2.3 Applied BioSystems) is used and signal normalization by U6. The relative expression levels of miRNAs is calculated using the comparative $\Delta\Delta$Ct method followed by log2-transformation. To identify longevity-associated miRNAs, the test of monotonicity based on non-parametric regression is applied, as proposed by Bowman et al. (111) and implemented in R-package sm (112). For those miRNAs that show significant non-monotonicity, a linear regression model is further fitted for subjects younger than 95 years old, and a t-test performed comparing those older than 95 with those between 80 to 90. Those miRNAs that show statistically significant negative slope in the linear regression model and show higher expression among centenarians (age >95) compared to those between 80-90 are selected for validation analysis. Similarly, those that show significant positive slope in the linear regression model and lower expression among centenarians compared to those between 80-90 are also selected for validation. In the data on miR-29c (Fig. 4C), a 1.6 standard deviation difference was observed between the 80-90 year old control groups and centenarians.

[0064] The validation study using expression data from LCLs and plasma is conducted in two independent analyses. First, for validation, in combined samples of controls and centenarians, the same test for monotonicity is performed as described previously. Second, expression levels of controls are compared with those of offspring. Under the standard framework of a linear model, it is tested if the slope of controls is different from offspring under the constraints that they have the same miRNA levels at age 50-60y. A miRNA is considered to be validated by cross-sectional data only if it shows statistical significance in both tests at significance of 0.05. Since the two tests are independent, the false positive rate for each miRNA is controlled at 0.052=0.0025, and is equivalent to control for overall Type-I error at 0.05 after Bonferroni correction, assuming 20 miRNAs are to be validated. In the test of monotonicity, based on simulation studies, it is estimated that if centenarians have the same miRNA expression levels as 65 years old controls, and at the age of 90, the controls are 0.3 standard deviations below the centenarians, an 85% power to detect this degree of non-monotonicity is available (Table 3).

[0065]

Table 3. Statistical Power for Validation Studies				
Analysis	Sample	Sig. Level	Detectable Difference	Power
1. Monotonicity Test	500 controls and 500 Cent. (Plasma or LCLs)	0.05	0.30 SD btw 90 yr controls and Cent.	0.85
2. Difference in Slopes	500 controls vs. 500 offspring (LCL/Plasma)	0.05	0.6(0.5) SD at 90 yr btw controls and offspring	0.9(0.8)

[0066] In the test of slope differences between offspring and controls, assuming the two groups have the same miRNA levels at age 65 but subsequently decline at different rates, a statistical power of 0.90 (and 0.80) is available to detect a difference in slopes that results in a 0.6 (and 0.5) standard deviation difference in mean miRNA levels at the age of 90 between the two groups (estimated using G*Power) (113). This cross-sectional analysis allows validation of longevity-associated miRNAs that show the maintenance of youthful expression patterns in centenarians and offspring. Estimates of "narrow sense' heritability (h2) can be made from the slope of linear regression of each parent on the mean value of offspring (114,83,115).

[0067] Data Analysis and Statistical Consideration. For binary phenotypes, a logistic regression model adjusted for age, sex, education, and other confounders is used and for continuous phenotypes, regular linear regression adjusted for age, sex, education, and other confounders is used to detect association. One miRNA and one target mRNA are considered at a time. Based on a linear regression model, at a significance level of 0.00083 (Bonferroni correction, 0.05/60), a statistical power of 0.80 is available to detect miRNA/mRNA that explains 4% of total variation in phenotypes. Multiple miRNA/mRNAs are entertained in the regression model together using model selection methods. Exhaustive searches can be performed, or otherwise Bayesian variable selection methods (142) are used. Finally, miRNA and mRNA expression results are used together with clinical phenotypes, and lifespan for causal modeling ('Mendelian Randomization') studies (143).

[0068] To confirm the targets of longevity-associated miRNAs, a luciferase reporter assay has been established using pMIR-REPORT vector (Ambion). Using this system, the interaction between miR-493 and its predicted target, eEF1A 3'UTR, was validated. The putative 3'UTR target site downstream of a luciferase reporter gene was cloned (Fig. 10A) and HeLa cells cotransfected with this vector together with miR-493 or the scrambled negative control (Ambion). Normalized luciferase activity of HeLa cells transfected with miR-493 was significantly decreased as compared to negative control (p= 0.003, t test, Fig.

10C). It was further tested whether the interaction between miR-493 and eEF1A1 3'UTR is direct or indirect by generating 2 mutations in the miR-493 predicted binding site in eEF1A1 3'UTR (Fig. 10B). Mutated eEF1A1 3'UTR was not regulated by miR-493 (Fig. 10C), demonstrating that eEF1A1 is a direct target of miR-493 through its binding to 3'UTR.

[0069] To identify all possible "real" targets, miRNA pull-down assay and CLIP technology (144,145) is used for 2-3 robust longevity-associated miRNAs. The causal relationship between longevity-associated miRNAs and reduced IGF1 signaling through down-regulation of key genes involved in this pathway can be determined utilizing established methods to measure IGF1-induced cell signaling, gene expression changes, cell cycle profiles, and stress resistance (1,2). Significant reverse correlations were found (Fig. 11A & 11B) in both expression levels and IGF1 signaling as measured by AKT phosphorylation after IGF1 treatment (1,2) between IGF1 and longevity-associated miRNAs predicted to target this gene in LCLs from a subset of centenarians who harbor longevity-associated miRNA signature (Fig. 3). While the reverse correlations in expression levels between IGF1 and longevity-associated miRNAs, e.g. miR-30b (Fig. 11C), from LCLs of all individuals were not as obvious as compared to a subset of centenarians with the longevity miRNA signature. These results suggest that down-regulation of IGF1 signaling through gene regulation by miRNA may in part contribute to longevity for a subset of centenarians.

Example 2

[0070] Functional role of longevity-associated miRNAs in modulation of conserved pathways of aging. MiRNAs alter cell and tissue phenotypes through alteration of target gene expression. To prioritize candidate miRNAs for comprehensive functional assays using an *in vitro* cell culture model, *in silico* prediction tools were used to identify targets genes and pathways of longevity-associated miRNAs as described (147). It was tested if target genes of longevity-associated miRNAs are part of known gene networks that impact on aging in general, using an online database and network analysis tool such as the NetAge database (148) and the Human Ageing Genomic Resources (HAGR) (149). Based on these predictions, a possible inverse-correlation was tested for in expression levels between a miRNA and its predicted target mRNAs by qPCR analysis measuring both "endogenous levels" in LCLs and regulated levels after overexpression using mimics or knock-down

using anti-miRs. For example, target sites of the 41 differentially expressed miRNAs in LCLs showed overrepresentation of genes involved in the insulin/IGF-1 (IIS) signaling pathway, the first and best characterized conserved pathway of aging. Reduced-function or reduced–expression of the components in the IIS pathway universally extends life span and delay the onset and progression of aging-related diseases in animal models. Whether the longevity-associated miRNAs target the conserved IIS pathway as reported in *C. elegans* was tested (150).

[0071] A negative correlation was demonstrated between the upregulated miRNAs in centenarians and the expression of IIS pathway genes by qPCR analysis, and IIS signaling as measured by phospho-AKT, in LCLs. To establish causal relationships between longevity-associated miRNAs and IIS, 10 miRNAs found to be upregulated in "both LCLs and plasma" of centenarians were overexpressed using MCF7 cells and HepG2 cells. MiR-142, miR-29b, miR-29c reduced IIS gene expression and signaling in MCF7 cells, while miR-142, miR-19a, miR-101 did so in HepG2 cells. MiR-142 had the largest impact on IIS in both cell lines.

[0072] Overexpression of miR-142 reduced: i) protein levels of IGF1R, INSR, RICTOR; ii) AKT phosphorylation at both S473 and T308 sites; iii) FOXO3 phosphorylation; and iv) mRNA levels of INSR, PI3KR2, RICTOR, and mTOR in MCF7 cells (Fig. 12). Luciferase reporter assays of fragments containing RICTOR 3'UTR predicted to bind miR-142 indicated that the second predicted site (707-713) is a likely target of miR-142 (Fig. 13 A and B). These results suggest that down-regulation of IIS and mTOR signaling genes by miR-142 may in part contribute to longevity. To identify all possible mRNA targets of miR-142, a pull-down assay using Bi-miR-142 (3′-biotinylated-miR-142) was used and confirmed BMAL1 (151) and RICTOR as its direct target (Fig. 13C).

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What is claimed is:

1. A method for determining if a subject is likely to develop an age-related disease comprising determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not likely to develop an age-related disease when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA.

2. A method for treating a subject for an age-related disease comprising determining if a subject is likely to develop an age-related disease comprising a) empirically determining the level of one or more of the following miRNAs in a sample obtained from the subject: miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a, and then comparing the levels of the miRNAs to predetermined control levels for each mRNA respectively, and identifying a subject as not suitable for treatment when the sample contains levels of the miRNAs above the respective predetermined control levels for each mRNA, and as suitable for treatment when the sample contains levels of the miRNAs below the respective predetermined control levels for each mRNA, and b) administering to a subject who has been identified as suitable for treatment in a) a treatment for an age-related disease, so as to thereby treat the subject.

3. The method of Claim 1 or 2, wherein when the sample contains levels of the miRNAs below the predetermined control levels for each mRNA, the subject is identified as likely to develop an age-related disease.

4. The method of Claim 1, 2 or 3, wherein the sample comprises plasma or cell-free serum.

5. The method of Claim 1, 2 or 3, wherein the sample comprises lymphoblastoid cells.

6. The method of any of Claims 1, 2, 3, 4, or 5, wherein a subject is identified as not likely to develop an age-related disease when all of miR-142, miR-101, miR-301b,

miR148a, miR21, miR-29c, miR30e, miR27a and miR15a are at a level above their respective predetermined control levels.

7. The method of any of Claims 1, 2, 3, 4, or 5, wherein a subject is identified as likely to develop an age-related disease when all of miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a are at a level below their respective predetermined control levels.

8. The method of any of Claims 1 or 3-7, further comprising testing a sample from a subject identified as likely to develop an age-related disease with a test predictive of development of, or predisposition to type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension, cognitive impairment, obesity, atherosclerosis, muscle atrophy or a neurodegenerative disease.

9. The method of any of Claims 1 or 3-7, further comprising treating a subject identified as likely to develop an age-related disease with a prophylactic treatment for an age-related disease.

10. The method of Claim 8, further comprising treating a subject identified as predisposed to, or likely to type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension or cognitive impairment with a treatment for type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension, cognitive impairment, obesity, atherosclerosis, muscle atrophy or a neurodegenerative disease, respectively.

11. A method for treating a subject for an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to treat an age-related disease in a subject.

12. A method for reducing the risk that a subject will suffer an age-related disease comprising administering to the subject an amount of an isolated miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a and miR15a effective to reduce the risk that a subject will suffer an age-related disease.

13. The method of Claim 11 or 12, wherein the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a is administered systemically.

14. The method of Claim 11, 12 or 13, wherein the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a is administered intravenously.

15. The method of Claim 11 or 12, wherein the miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a administered is a locked nucleic acid miR-142, miR-101, miR-301b, miR148a, miR21, miR-29c, miR30e, miR27a or miR15a.

16. The method of any of Claims 11-15, wherein the miR-142 is administered.

17. The method of any of Claims 11-16, wherein the microRNA administered has the same sequence as a corresponding human microRNA.

18. The method of any of Claims 1-17, wherein the age-related disease is type II diabetes, metabolic syndrome, a cardiovascular disease, hypertension or cognitive impairment.

19. The method of any of Claims 1-17, wherein the age-related disease is cardiovascular disease and is stroke, myocardial infarction, or a coronary vascular disease.

20. The method of any of Claims 1-19, wherein the subject is a human subject.

21. The method of Claim 16, wherein the amount of miR-142 administered is sufficient to decrease IGF1 signaling in a subject.

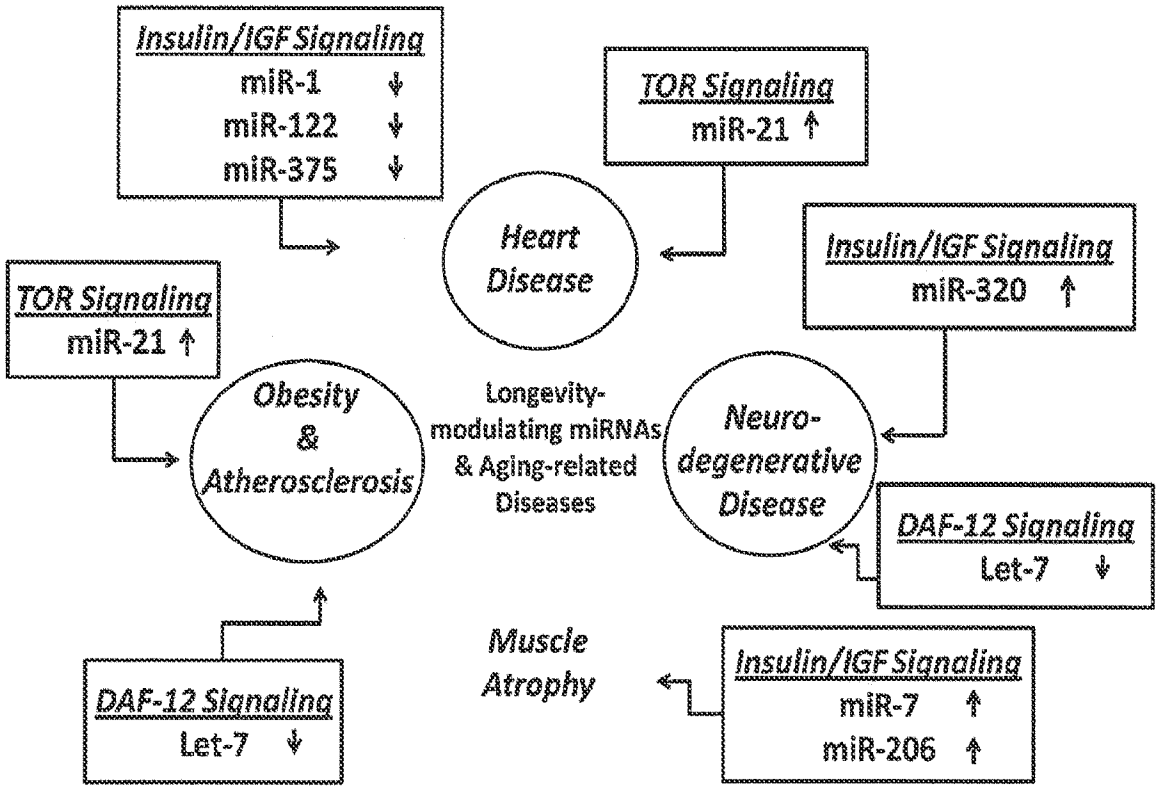

Insulin/IGF Signaling
miR-1 ↓
miR-122 ↓
miR-375 ↓
TOR Signaling
miR-21 ↑
Heart Disease
TOR Signaling
miR-21 ↑
Insulin/IGF Signaling
miR-320 ↑
Obesity & Atherosclerosis
Longevity-modulating miRNAs & Aging-related Diseases
Neuro-degenerative Disease
DAF-12 Signaling
Let-7 ↓
DAF-12 Signaling
Let-7 ↓
Muscle Atrophy
Insulin/IGF Signaling
miR-7 ↑
miR-206 ↑

Fig. 1

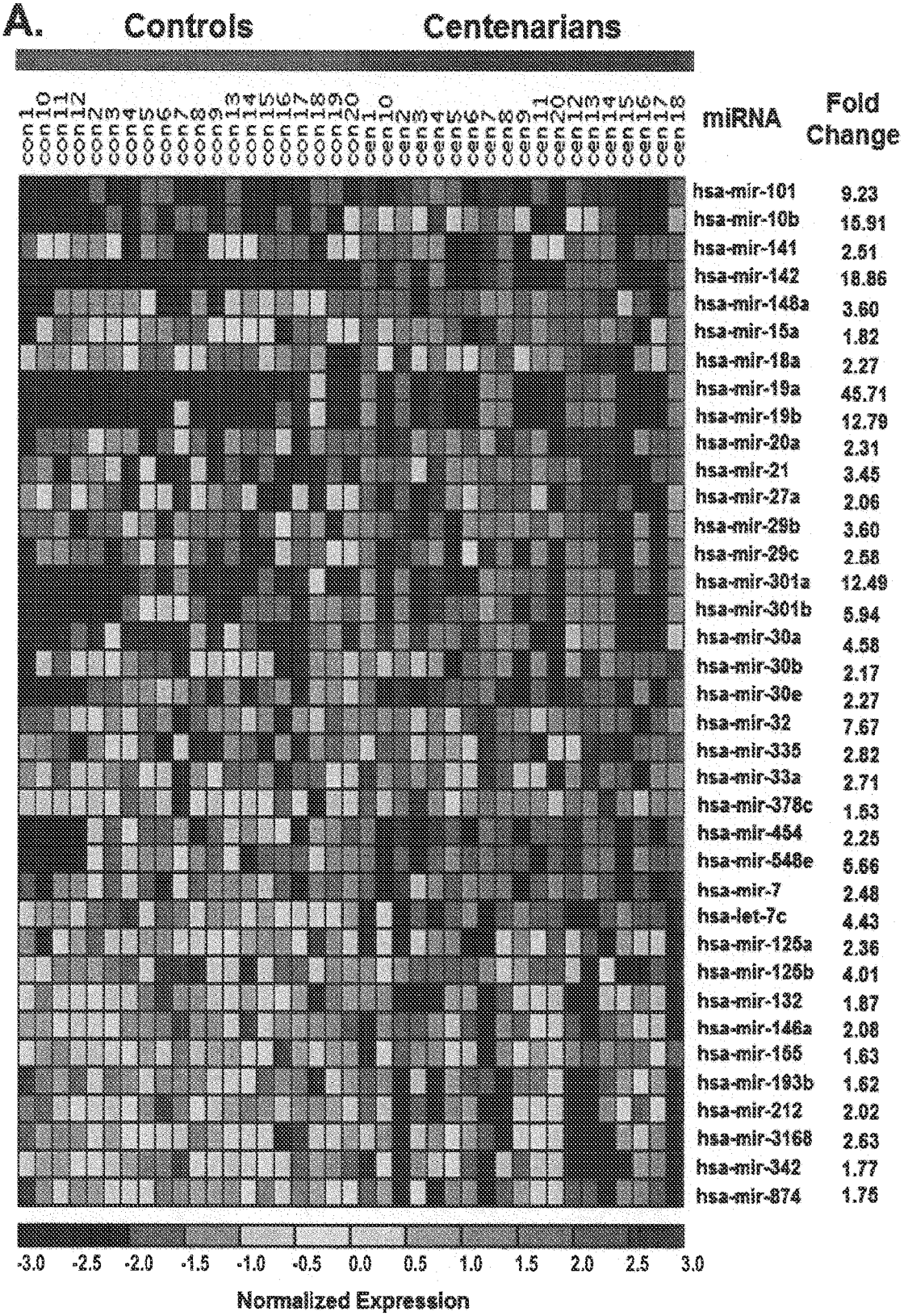
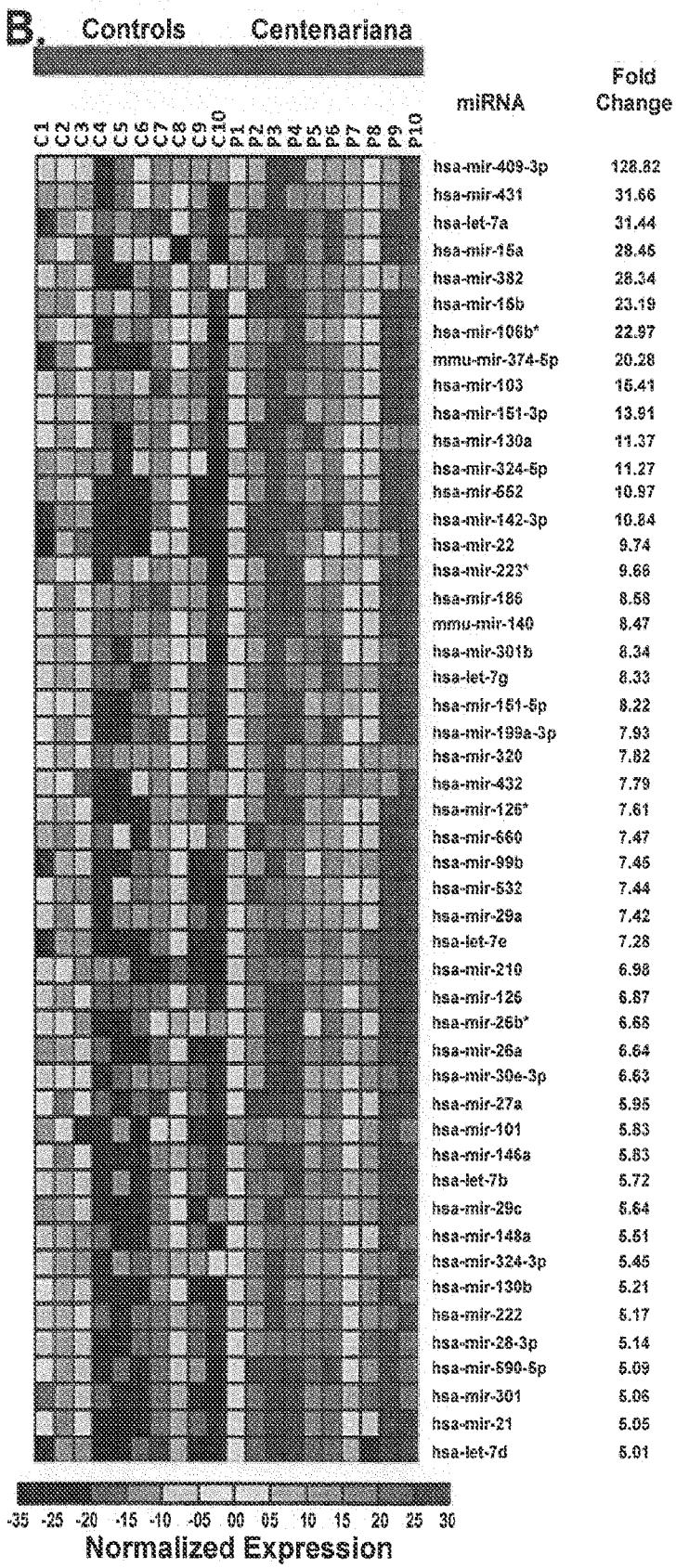

A.
Controls
Centenarians
con1 con10 con11 con12 con2 con3 con4 con5 con6 con7 con8 con9 con13 con14 con15 con16 con17 con18 con19 con20
cen1 cen10 cen2 cen3 cen4 cen5 cen6 cen7 cen8 cen9 cen11 cen12 cen13 cen14 cen15 cen16 cen17 cen18
miRNA
Fold Change
hsa-mir-101 9.23
hsa-mir-10b 15.91
hsa-mir-141 2.51
hsa-mir-142 18.86
hsa-mir-148a 3.60
hsa-mir-15a 1.82
hsa-mir-18a 2.27
hsa-mir-19a 45.71
hsa-mir-19b 12.79
hsa-mir-20a 2.31
hsa-mir-21 3.45
hsa-mir-27a 2.06
hsa-mir-29b 3.60
hsa-mir-29c 2.58
hsa-mir-301a 12.49
hsa-mir-301b 5.94
hsa-mir-30a 4.58
hsa-mir-30b 2.17
hsa-mir-30e 2.27
hsa-mir-32 7.67
hsa-mir-335 2.82
hsa-mir-33a 2.71
hsa-mir-378c 1.53
hsa-mir-454 2.25
hsa-mir-548e 5.66
hsa-mir-7 2.48
hsa-let-7c 4.43
hsa-mir-125a 2.36
hsa-mir-125b 4.01
hsa-mir-132 1.87
hsa-mir-146a 2.08
hsa-mir-155 1.63
hsa-mir-183b 1.62
hsa-mir-212 2.02
hsa-mir-3168 2.63
hsa-mir-342 1.77
hsa-mir-874 1.75
-3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0
Normalized Expression

Fig. 3A



miRNA	Fold Change
hsa-mir-409-3p	128.82
hsa-mir-431	31.66
hsa-let-7a	31.44
hsa-mir-15a	28.45
hsa-mir-382	28.34
hsa-mir-15b	23.19
hsa-mir-106b*	22.97
mmu-mir-374-5p	20.28
hsa-mir-103	15.41
hsa-mir-151-3p	13.91
hsa-mir-130a	11.37
hsa-mir-324-5p	11.27
hsa-mir-652	10.97
hsa-mir-142-3p	10.84
hsa-mir-22	9.74
hsa-mir-223*	9.66
hsa-mir-186	8.58
mmu-mir-140	8.47
hsa-mir-301b	8.34
hsa-let-7g	8.33
hsa-mir-151-5p	8.22
hsa-mir-199a-3p	7.93
hsa-mir-320	7.82
hsa-mir-432	7.79
hsa-mir-126*	7.61
hsa-mir-660	7.47
hsa-mir-99b	7.45
hsa-mir-532	7.44
hsa-mir-29a	7.42
hsa-let-7e	7.28
hsa-mir-210	6.98
hsa-mir-126	6.87
hsa-mir-26b*	6.68
hsa-mir-26a	6.64
hsa-mir-30e-3p	6.63
hsa-mir-27a	5.95
hsa-mir-101	5.83
hsa-mir-146a	5.83
hsa-let-7b	5.72
hsa-mir-29c	5.64
hsa-mir-148a	5.51
hsa-mir-324-3p	5.45
hsa-mir-130b	5.21
hsa-mir-222	5.17
hsa-mir-28-3p	5.14
hsa-mir-590-5p	5.09
hsa-mir-301	5.06
hsa-mir-21	5.05
hsa-let-7d	5.01

Fig. 3B

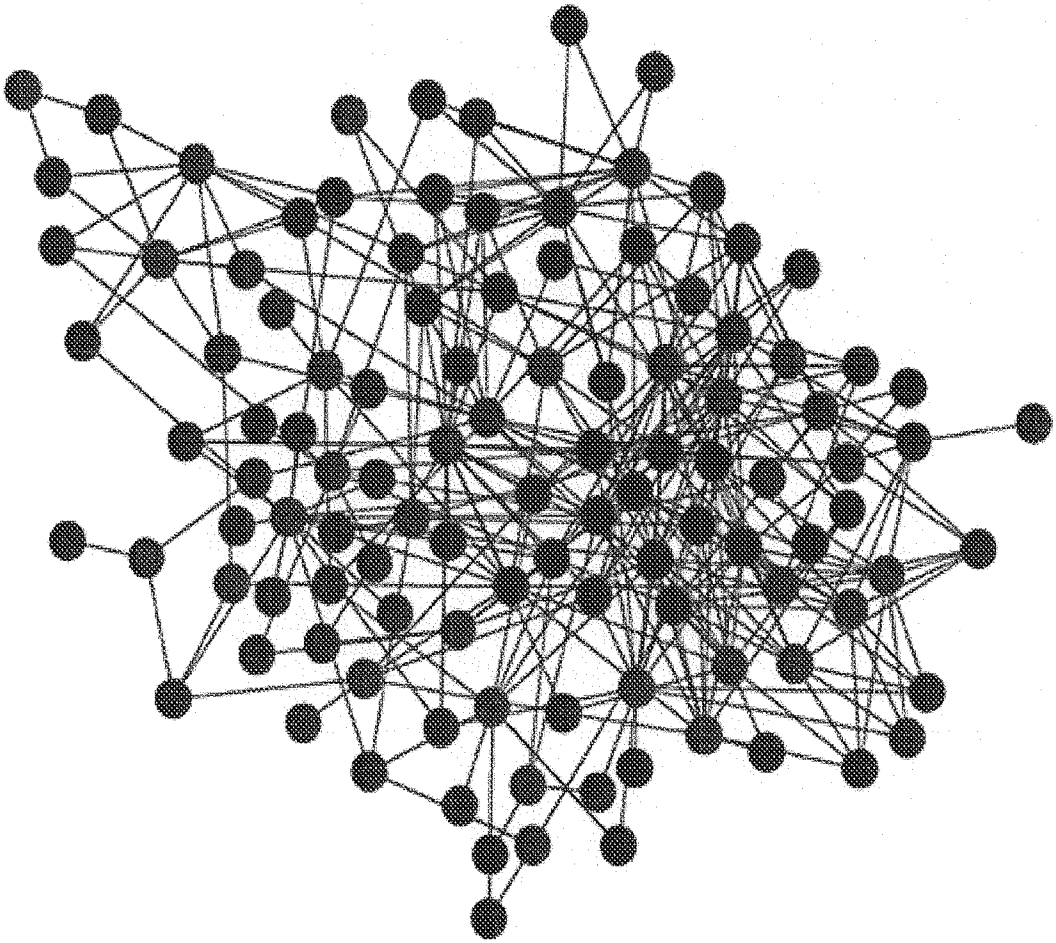
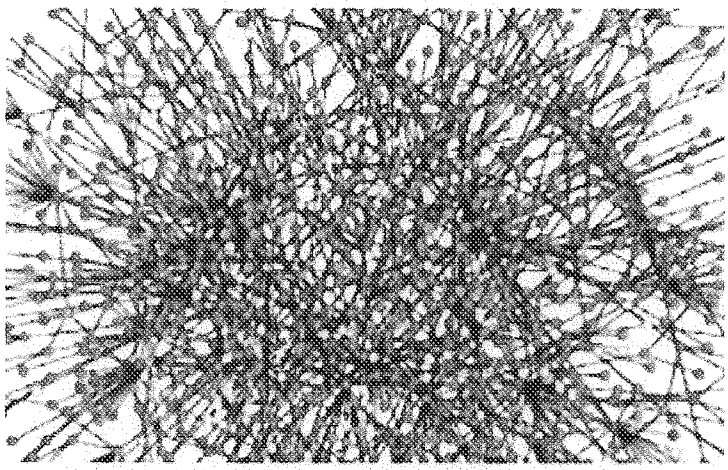
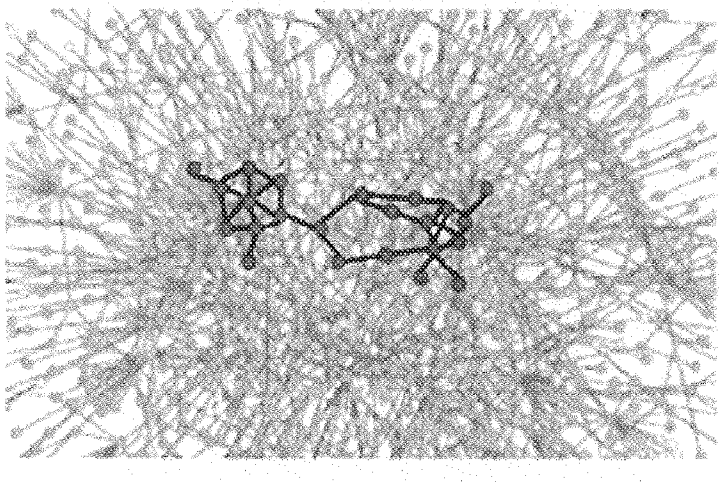


Fig. 6

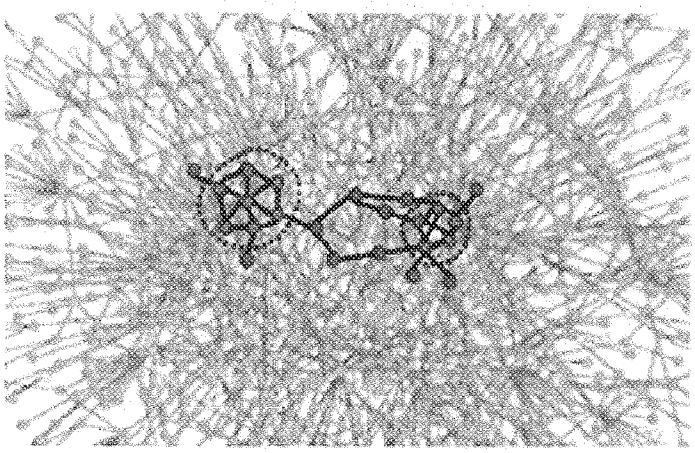
A)



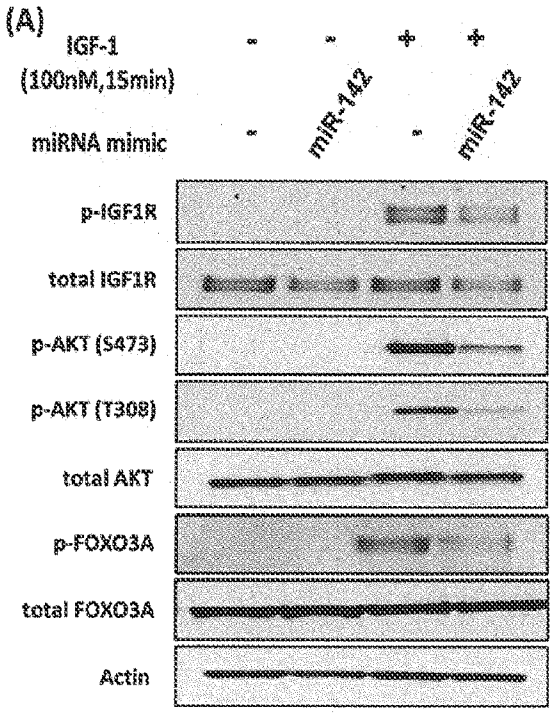
B)



C)



9A-C


(A)
IGF-1
(100nM,15min)
miRNA mimic
-
-
+
+
-
miR-142
-
miR-142
p-IGF1R
total IGF1R
p-AKT (S473)
p-AKT (T308)
total AKT
p-FOXO3A
total FOXO3A
Actin

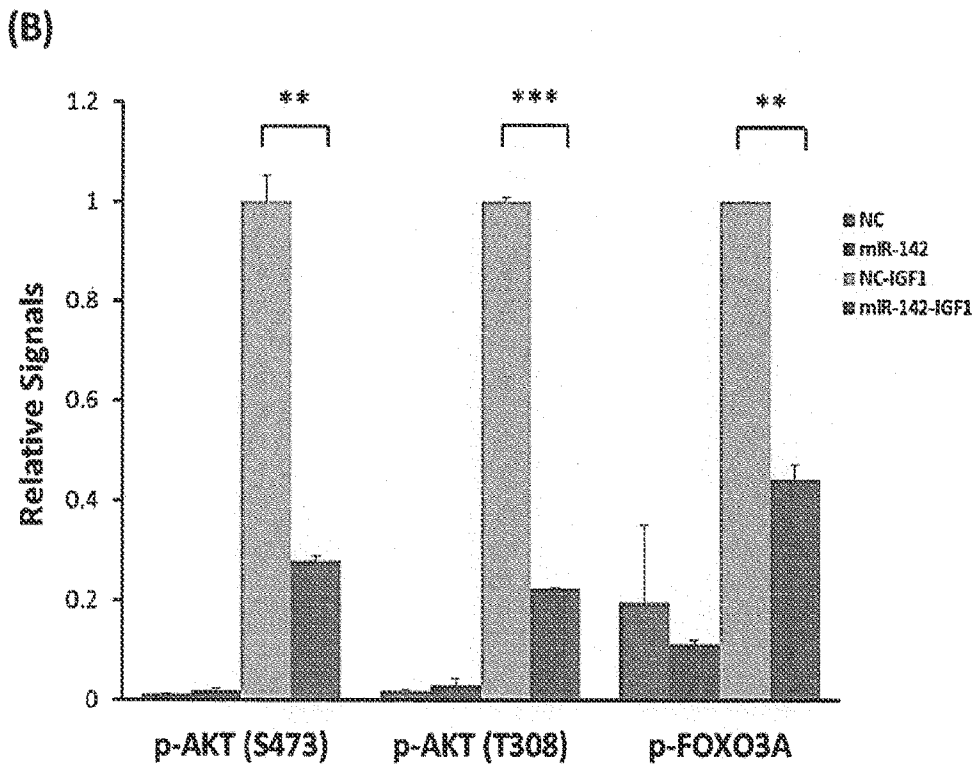
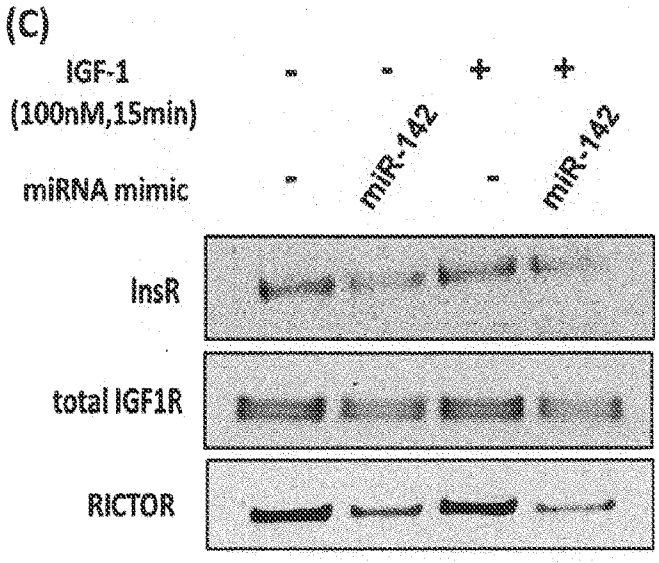

(B)
Relative Signals
1.2
1
0.8
0.6
0.4
0.2
0
**
***
**
NC
miR-142
NC-IGF1
miR-142-IGF1
p-AKT (S473)
p-AKT (T308)
p-FOXO3A

Fig. 12A-B


(C)
IGF-1
(100nM,15min)
miRNA mimic
-
-
+
+
-
miR-142
-
miR-142
InsR
total IGF1R
RICTOR

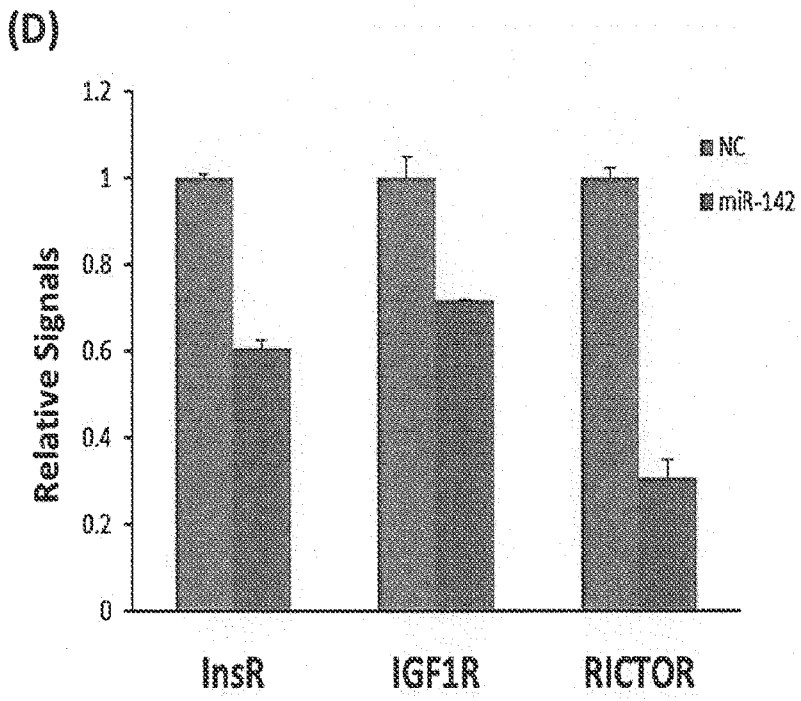

(D)
Relative Signals
1.2
1
0.8
0.6
0.4
0.2
0
NC
miR-142
InsR
IGF1R
RICTOR

Fig. 12C-D

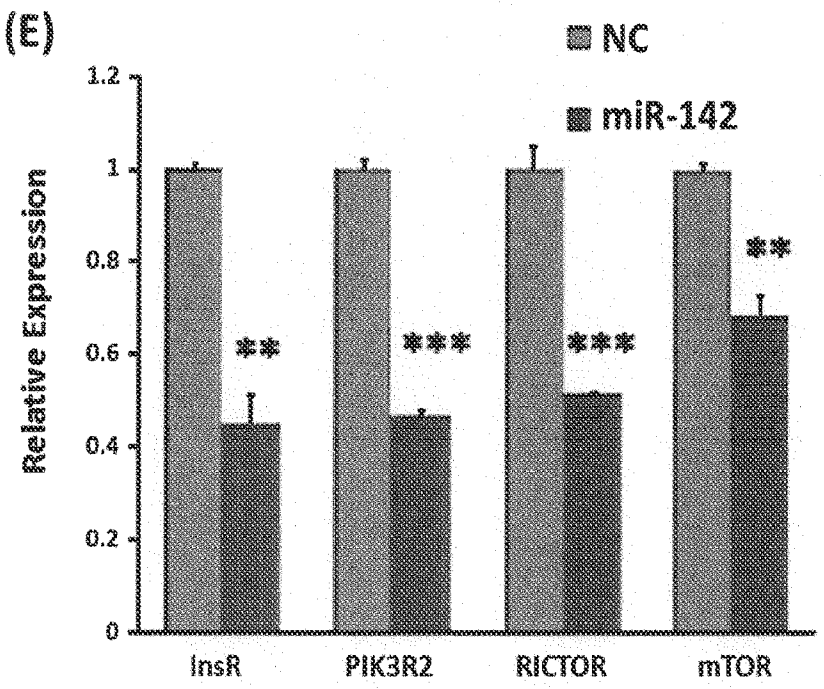
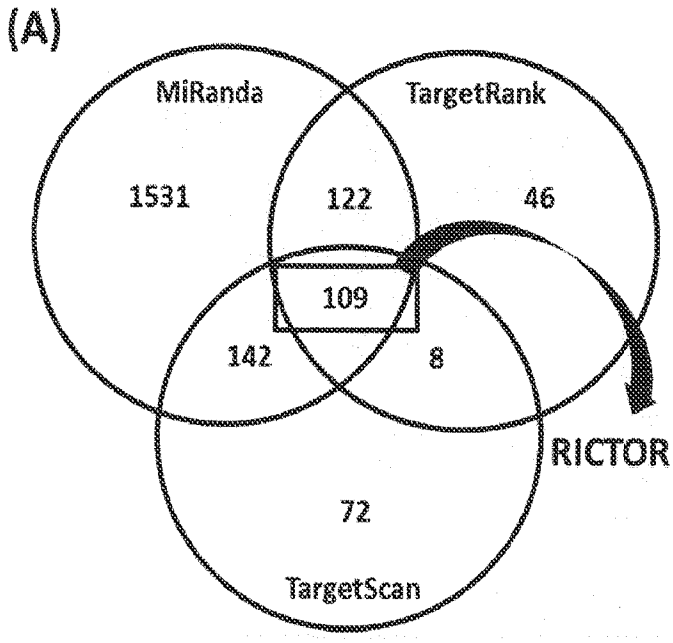
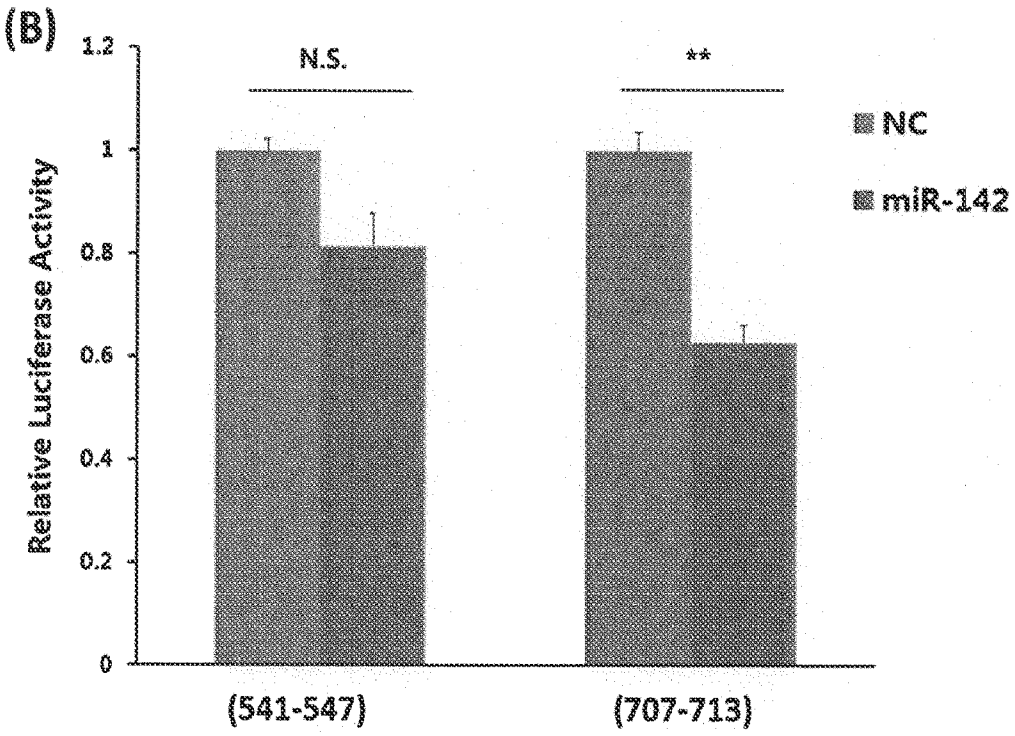

(E)
NC
miR-142
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
**
***
***
**
InsR
PIK3R2
RICTOR
mTOR

Fig. 12E


(A)
MiRanda
TargetRank
1531
122
46
109
142
8
RICTOR
72
TargetScan


(B)
N.S.
**
NC
miR-142
Relative Luciferase Activity
1.2
1
0.8
0.6
0.4
0.2
0
(541-547)
(707-713)

Figs. 13A-B

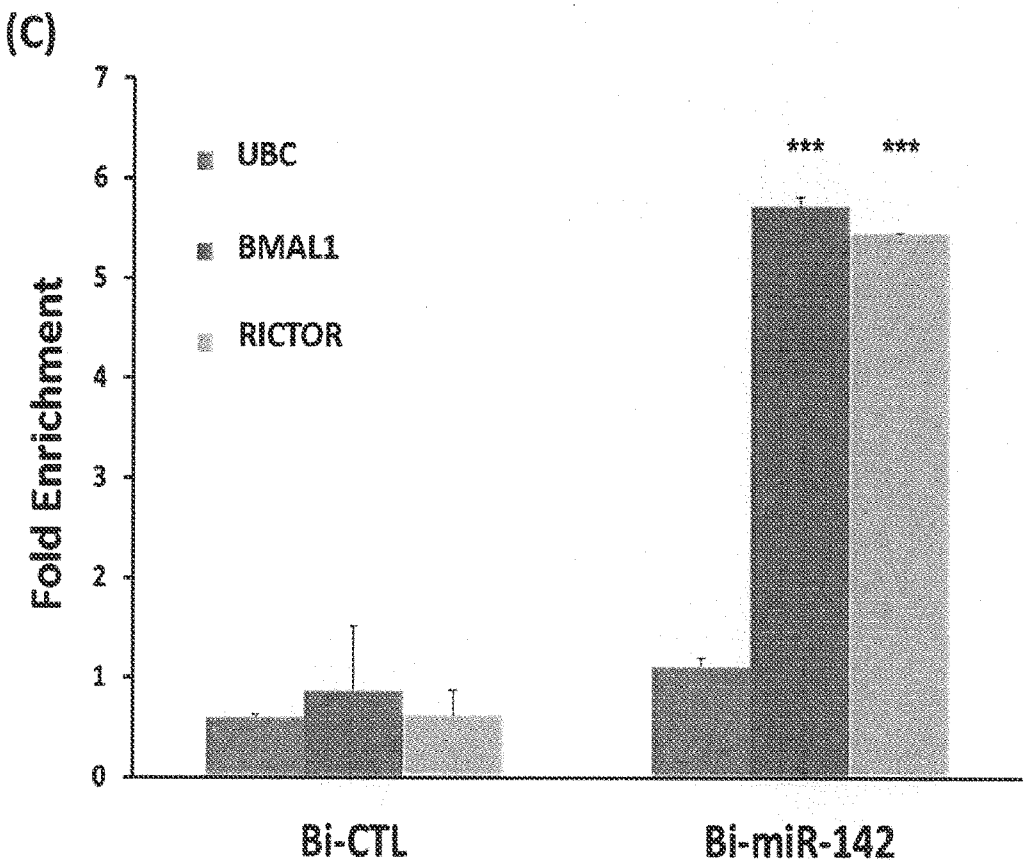

(C)
Fold Enrichment
0
1
2
3
4
5
6
7
UBC
BMAL1
RICTOR
***
***
Bi-CTL
Bi-miR-142

Fig. 9C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/27113

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; C12P 19/34 (2014.01)
USPC - 435/6.1, 4, 91.1, 89, 85, 84, 72, 41
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)
IPC(8): C12Q 1/68, 1/02; C12P 19/34; C40B 40/06, 30/04; G01N 33/48, 33/53, 27/447; C12N 15/113, 5/09, 15/85 (2014.01)
USPC: 435/6.1, 4, 91.1, 89, 85, 84, 72, 41, 6.11, 375, 7.92, 7.1, 455; 506/9, 16, 7; 514/44A, 327; CPC Classification(s): C12Q 1/6844

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; GoogleScholar; ProQuest; administering, 'miRNA,' treating

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	DONG, S. et al. MicroRNA Expression Signature And The Role Of MicroRNA-21 In The Early Phase Of Acute Myocardial Infarction. The Journal of Biological Chemistry. 25 August 2009, Vol. 284; pages 29514-29525; abstract; page 29515, right column, third paragraph; page 29518, left column, third paragraph; page 29518, figures 2A, 2B; page 29518, right column, second paragraph; page 29518, figure 2C; page 29519, figures 3B, 3C.	1, 2, 3/1, 3/2, 11, 13/11 — 12, 13/12, 15/11, 15/12
Y	US 2012/0065248 A1 (BROWN, D et al.) 15 March 2012; paragraph [0100]	12, 13/12, 15/12
Y	LIU, G et al. miR-21 Mediates Fibrogenic Activation Of Pulmonary Fibroblasts And Lung Fibrosis. The Journal of Experimental Medicine. 19 July 2010, Vol. 207, No. 8; pages 1589-1597; page 1590, right column, third paragraph.	15/11, 15/12
A	LI, Y et al. MicroRNAs In Common Human Diseases. Genomics Proteomics & Bioinformatics. 29 September 2012, Vol. 10; pages 246-253; entire document.	1, 2, 3/1, 3/2, 11, 12, 13/11, 13/12, 15/11, 15/12

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2014 (12.08.2014)	27 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/27113

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

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

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

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

3. ☒ Claims Nos.: 4-10, 14, 16-21
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 additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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.