Abstract:
The present invention relates to the identification of a number of miRNAs that are up-regulated in expression in aged cells, tissues, organs and organisms. The expression level of the miRNAs was especially higher in aged liver of normally and healthy aged mice. When ageing was slowed down through dietary restriction, the expression levels dropped as compared to non-treated animals. These miRNAs, alone or in combination provide a useful tool in diagnostics and research in the field of ageing and/or the treatment or prevention of disorders and features related to increased age.

Title: USE OF MICRONRNAS IN DIAGNOSIS AND THERAPY OF AGING

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Title: Use of microRNAs in diagnosis and therapy of aging

FIELD OF THE INVENTION

The invention relates to the field of medicine and in particular to the field of preventing and/or treating ageing-related diseases. More in particular the invention relates to the field of DNA damage repair, the identification of microRNA (miRNA) and their role in DNA damage response and the interface with the process of ageing and the use of miRNAs (and their expression levels) in diagnostics and/or as a research tool and/or a means to intervene.

BACKGROUND OF THE INVENTION

The cellular DNA in each living cell in the mammalian body is continuously attacked and damaged by various endogenous and exogenous agents, such as metabolic byproducts and UV radiation. Generally, said DNA damage is repaired correctly by the cellular repair systems. However, when not repaired properly this DNA damage can result in mutations or chromosomal aberrations and eventually cause cell death and cellular senescence or trigger the appearance of severe disorders such as cancer. Mammalian cells have elaborate systems to counteract the potential harmful effects of DNA damage, collectively referred to as 'DNA damage response' or in the case that such damage is being repaired: 'DNA damage repair'. The appearance of DNA damage may activate different signal transduction routes, it may halt the cell cycle, and it may trigger apoptosis or lead to irreversible growth arrest. In parallel, DNA damage generally triggers different kinds of repair systems. Precise and accurate regulation of the repair is critical for cell- and organism survival. Its abrogation or malfunction may on the one hand cause mutations (permanent alterations in the genetic code) when damaged DNA is still used as template for replication in proliferating cells. On the other hand inadequate repair may lead to cell death or permanent cell cycle arrest (cellular senescence) which in the end may cause exhaustion of the cell renewal capacity of the organ or tissue, loss of homeostasis, hypocellularity,
tissue malfunction and in this manner contribute to aging (Hoeijmakers, NEJM, 2009). It is known that expression, stability and functionality of proteins involved in DNA damage repair can be regulated at the post-translational level. DNA damage repair is also controlled at the transcriptional level by the induction of cell cycle regulatory genes after genotoxic stress. The exact processes and the possibilities to control and influence the level of activity and expression of proteins involved in DNA damage repair have been the subject of intense investigations, since detailed knowledge of the function and dysfunction of DNA damage repair mechanisms would provide tools to treat disorders caused by aberrant DNA damage responses. It is believed that an increased level of non-repaired DNA in the cells contributes to the occurrence of many disorders that appear more frequently during the process of ageing, such as cancer, cardiovascular diseases, osteoporosis, neurodegeneration, and amyloid plaque forming-related diseases such as Alzheimer's Disease or diabetes type 2. A proper understanding of the organization of DNA damage repair and the control of the genetic processes underlying these responses, including the knowledge of the pathways and regulation of protein expressions would provide insight in the occurrence of such diseases and potentially provide the means to inhibit, prevent or cure such DNA damage-related disorders. There is a general need for such understanding, but also for proper tools to measure and determine the level of severity in such ageing-related processes and progressive disorders in the course of such research.

Gene regulation and protein expression have been studied for many decades. Signal transduction pathways, the role of transcription factors, promoters, enhancers, co-activators, co-repressors, and protein complexes involved in transcription control and RNA stability, etc. have been extensively investigated and many processes have been elucidated over the years. Recently, an additional layer of gene regulation has been discovered, which acts at the post-transcriptional level through so-called microRNAs (herein also
referred to as miRNAs). miRNAs comprise an abundant class of small non-coding endogenous RNAs (~22 nucleotides) that bind to partially complementary sites within the 3'-UTR's of target mRNAs, and post-transcriptionally modulate gene expression by facilitating translational repression or mRNA degradation. They are found to act in diverse cellular and developmental processes in microorganisms, plants and animals. Mature miRNAs are generated from longer primary transcripts that are processed in the nucleus into hairpin RNAs of ~70 nucleotides. These precursor miRNAs are exported to the cytoplasm and further processed to mature miRNAs.

Silencing by miRNAs is accomplished by the RNA-Induced Silencing Complex (RISC), which comprises the miRNA together with a number of proteins including the Argonaut (Ago) proteins.

It has been estimated that as many as 1000-2000 miRNA genes are encoded in the mammalian genome, and to date approximately 900 human miRNAs have been experimentally verified. Most of the miRNAs and their target sites are highly conserved between species. Approximately 30% of the protein-coding genes contain potential miRNA binding sites in their 3'-UTR and are thought to be under direct control (via degradation) of miRNAs.

The idea has been raised that miRNAs play a role in ageing-related disorders such as cancer and Alzheimer's Disease, and in the control of ageing or life-span. It has been reported that the miRNA derived from the lin-4 gene in C. elegans is involved in ageing control by inhibiting the expression of the lin-14 mRNA and lin-14 protein causing enhanced expression of the DAF-16 transcription factor and promoting long life. It has also been shown (WO 2007/002528) that lin-14 miRNA can downregulate genes involved in senescence, lifespan, or age-related disorders. miRNAs most clearly contribute to regulation of cell cycle checkpoints and apoptosis, but may also influence other aspects of cellular metabolism, differentiation and proliferation. It has been found that miRNA-mediated gene silencing modulates UV-induced DNA-damage responses (Pothof et al. 2009).
Ageing is a fact of life, and numerous diseases and disorders occur more frequently as the organism ages. Scientific research has more than often focused on ways to prevent, cure, treat or diagnose such diseases. Curing or treating ageing-related disorders is the subject of intense studies. Although the ultimate goal is survival or prolonging life, such research is often hampered by the lack of quick, reliable and useful tools to determine whether a certain treatment is effective. The inventors of the present invention have sought to find (diagnostic) tools based on miRNA's and the role of miRNA's in DNA damage control (pathways) and through this, the role of miRNA's in ageing.

SUMMARY OF THE INVENTION

The present invention provides a method for determining whether a subject, a tissue or a cell exhibits an ageing genotype, the method comprising:
- providing a sample from said subject;
- determining in said sample the amount of the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451;
- comparing said amount with a reference amount (acting as internal reference); and
- determining from said comparison whether or not the subject, a tissue or a cell exhibits an ageing genotype.

The present invention provides a method for determining an ageing stage of a subject, a tissue or a cell, the method comprising:
- providing a sample from said subject or tissue or a cell;
- determining in said sample the amount of the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an advanced ageing stage selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-
- comparing said amount with a reference amount (acting as internal reference); and

- determining from said comparison whether or not in the subject, tissue or cell said miRNAs are upregulated relative to said reference.

In a method of the present invention as described above, the amount of the pri-miRNA and/or pre-miRNA and/or miRNAs is determined in the form of a miRNA profile for miRNAs let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, preferably miRNAs let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193, still more preferably miRNAs miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; and miR-29b; miR-30a-5p; and miR-30e.

The present invention further provides a kit of parts adapted for use in a method according to the present invention as described above, said kit comprising:

- at least one compound capable of specifically binding the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451; and

- instructions for use of said compound for determining whether a subject, a tissue or a cell exhibits an ageing genotype.

The present invention further provides a method for determining whether a candidate compound is capable of counteracting, treating,
diminishing, delaying and/or preventing an ageing genotype or a disorder associated with ageing, the method comprising:

- contacting said candidate compound with a cell, tissue or subject, preferably with a liver, kidney, spleen or lung cell, and

- determining whether said candidate compound is capable of silencing one, preferably all, miRNAs selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, or determining whether said candidate compound is capable of preventing or counteracting the decrease in protein expression associated with the upregulation of said miRNAs observed in an ageing genotype.

In a preferred embodiment of this method, the method further comprises the steps of:

- assaying the survivability of the cell, tissue or subject contacted with the candidate compound or the severity of the disorder;

- comparing the survivability or the severity of the disorder of the cell, tissue or subject contacted with the test compound to a control cell, tissue or subject; and

- selecting the compound that prolongs the survivability or ameliorates (the effects of) the disorder of the cell, tissue or subject contacted with the test compound compared to survivability of the control cell, tissue or subject.

The present invention further provides a method for counteracting, treating, diminishing, delaying and/or preventing disorders associated with the ageing genotype, in a subject, the method comprising:

- decreasing the expression, amount and/or activity of a miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, within said subject, and/or
- decreasing the interaction of the specific target mRNA with a miRNA selected from the group consisting of from selected from the group consisting of let-7c; let-7a; let-7f; miE-16; miR-21; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, within said subject.

In a preferred embodiment of this method, the age-related disorder is selected from the group consisting of Alzheimer's, Parkinson's, diabetes, dementia, atherosclerosis, arthritis, stroke, high blood pressure, and heart disease.

In a further preferred embodiment of this method, the subject is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. microRNA expression in aged mice. A. unsupervised hierarchical clustering of 4 tissues (kidney, lung, liver and spleen) from male C57BL/6 mice at 3 ages (13 weeks, 52 weeks and 104 weeks) using the average from n=3 per organ/time point. MicroRNAs expressed at background levels were excluded.

B. heatmap of 14 significantly, commonly regulated microRNAs across liver, lung, spleen and kidney from aged male C57BL/6 mice. Fold change compared to 13 week old organs is shown. Heatmaps of common set of 14 microRNAs in:

C. 130 week old livers from male C57BL/6 mice.

D. DR male C57BL/6 mice. 20 week old mice were fed ad libitum or underwent DR (dietary restriction) for 6 weeks. Fold change between DR and ad libitum fed animals is shown.

E. livers from 4 and 17 week old ERCC1^/- male mice. Fold change is shown compared to age-matched wild type littermate controls.

F. MEFs 8 hours after shifting from 3% O_2 to 20% O_2. Fold change is shown compared to primary mouse embryonic fibroblasts grown at 3% O_2. NIH3T3 cells 4 hours after H2O2 (100 µM) or ionizing radiation (6 Gy) treatment. Fold change is shown compared to mock-treated cells.

G. NIH3T3 cells and human dermal fibroblasts 4 hours after UV-C irradiation (8J/m2). Fold changes compared to mock-treated cells is shown. Red color indicates upregulation, blue down-regulation.
**Figure 2.** MicroRNA target gene predictions. **A.** Panel I: percentage of significantly regulated genes from 2.5 year old male C57BL/6 livers that are predicted microRNA target genes against 1) all significantly regulated microRNAs in 2.5 year old livers, 2) the 14 commonly regulated microRNAs, and 3) all overlapping microRNAs between 2.5 year old livers and UVC treated NIH3T3 cells. Panel II: percentage of significantly regulated genes from 300 most downregulated genes across 22 tissues in mouse aging that are predicted microRNA target genes against the 14 common regulated microRNAs. Five similar sized random gene sets were used as control. Overrepresentation was calculated by Fisher Exact test (**\( \rightarrow p<0.001 \)). **B.** The direction of gene expression regulation in gene expression arrays from UV-C treated NIH3T3 cells (8 J/m2; 4 hours after treatment) was determined for: panel I) significantly regulated genes from 130 week old livers that are predicted targets of overlapping microRNAs between UVC treated NIH3T3 cells and 130 week old livers (microRNA set 3, Figure 2A, panel I). Only significant Affymetrix probe IDs from 130 week old livers were matched. Panel II) the 300 most down-regulated genes across 22 tissues in mouse aging that are predicted target genes of overlapping microRNAs between the 14 commonly regulated microRNAs and UV-C treated NIH3T3 cells. Since specific Affymetrix probe IDs were not specified, either one specific probe ID for a gene was used when significantly regulated (SAM analysis; FDR<5%) or the average fold change from all probe IDs together for the same gene. Panel III represents the direction of regulation of all probe IDs within the gene expression arrays from UVC-treated NIH3T3 cells. DAVID analysis using KEGG and Biocarta pathways of: **C.** all significantly regulated genes from 2.5 year old livers. **D.** all predicted gene targets in 2.5 year old C57BL/6 livers from all significantly regulated microRNAs in 2.5 year old C57BL/6 livers (Figure 2A, microRNA set 1). **E.** all predicted gene targets in 2.5 year old C57BL/6 livers from the overlapping microRNAs between 2.5 year old livers and UVC-treated NIH3T3 cells (Figure 2A, microRNA set 3).
Figure 3. PTEN is a miR-26 target gene. A. schematic overview of PI3 kinase signaling. B. Quantitative RT-PCR for PTEN in indicated tissues. Tubulin is used as reference gene. * p<0.05, student's t-test. C. Full length PTEN 3'UTR is cloned downstream Renilla luciferase and transiently co-transfected with miR-26a mimicking oligonucleotide in HEK293T cells (upwards arrow: up-regulation). Empty pSICHECK II and non-targeting mimicking oligonucleotide were used as controls. 24 hours later cells were lysed and used for bioluminescence measurements. ** p<0.001, student's t-test. D. Immuno-blotting of NIH3T3 cells transfected with either miR-26a mimicking oligonucleotide or control mimicking oligonucleotide (upwards arrow: up-regulation). E. Quantitative RT-PCR of PTEN and the reference gene Tubulin in NIH3T3 cells that were transfected with either combined miR-26a and miR-26b antisense microRNA oligonucleotides or a control antisense oligonucleotide (downwards arrow: down-regulation) and exposed to UVC (8J/m2). 4 hours later total RNA was isolated. * p<0.05, student's t-test. F. Immunoblotting of NIH3T3 cells that were transfected with either a miR-26a mimicking oligonucleotide or a control (upwards arrow indicates up-regulation) and serum deprived for 24 hours followed by insulin stimulation and for the indicated time points. G. Heatmap of FOXO3a target genes, which are at least significantly regulated in 2.5 year old livers, 17 week ERCC1^-/- livers or after UVC irradiation as seen in their respective Affymetrix gene expression arrays. The first column represents the direction of gene regulation when FOXO transcription factors are activated. Blue color represents decreased expression, red represents increased expression. H. Real-time luciferase monitoring was performed in NIH3T3 cells that were co-transfected with a vector containing a luciferase driven by 6x DBE synthetic promoter element and either combined miR-26a and miR-26b antisense microRNA oligonucleotides or a control antisense oligonucleotide. Cells were UV-C irradiated (8J/m2) or mock-treated. I. Cell survival by miR-26 and PTEN. NIH3T3 cells were transfected with the indicated siRNAs, miR-26a mimicking oligonucleotides or miR-26a and miR-
26b antisense microRNA oligonucleotides. Upwards arrows indicate miR-26a
upregulation, downward arrows down-regulation of gene/microRNA. 24 hours
after transfection, cells were put at 0.5% serum and 24 hours later irradiated
with UVC (12J/m²). 24 hours later, the number of living cells was counted. * p<0.05, ** p<0.001, Student's t-test.

**Figure 4. NRF2 activation via miR-26.** A. Heatmap of NRF2 target
genes, which are at least significantly regulated in 130 week old livers, 17
week ERCC1^5/-livers or after UVC irradiation as seen in their respective
Affymetrix gene expression arrays. Blue color represents decreased expression,
red represents increased expression. B. Immunoblotting of NIH3T3 cells that
were transfected with either a miR-26a mimicking oligonucleotide or a control
(upwards arrow indicates up-regulation) and treated with LY294002. C. Distribution of GFP-NRF2 after UV-C treatment. NIH3T3 cells were co-
transfected with GFP-NRF2 and the indicated siRNAs, miR-26 mimicking
oligonucleotide, miR-26a and 26b antisense microRNA oligonucleotide or a
combination thereof. Upwards arrows indicate miR-26a up-regulation,
downwards arrows down-regulation of gene/miR-26a and 26b. Panel I contains
3 representative pictures of GFP-NRF2 expressing cells and the estimated
level of expression in nucleus and cytoplasm: I.I mostly cytoplasmatic or
equally distributed between cytoplasm and nucleus, I.II mostly nuclear, I.III
highly enriched in the nucleus. The percentage cells with specific GFP-NRF2
expression distribution is quantified before (panel II) and 4 hours after UV-C
treatment (8J/m²) (panel III). D. and E. Protective effect of UV-C against H2O2
by NRF2. NIH3T3 cells were transfected with the indicated siRNAs, miR-26
mimicking oligonucleotide, miR-26a and 26b antisense microRNA
oligonucleotide (D) or a combination thereof (E). Upwards arrows indicate
upregulation of miR-26a, downwards arrow down-regulation of gene/ miR-26a
and 26b. 8 hours after UV-C irradiation (8J/m²) cells were treated with 100
µM H2O2. The next day the number of living cells was counted. * p<0.05, **
p < 0.001, Student’s t-test. 

**Figure 5.** Signature microRNA regulation in delayed and accelerated aging and after cellular stresses. This figure shows the aging genotype profile as described herein.

**Figure 6.** Statistics and selection criteria for determining microRNA signature across liver, lung, kidney spleen. MicroRNAs were identified as commonly regulated between 13 week and 104 week old tissues, when I) False Discovery Rate (FDR) < 5% in a 2-class paired Statistical Analysis of Microarrays (SAM) between the average 13 week and average 104 week of each organ and a minimal fold change of >1.5 or <-1.5, II) FDR < 5% using SAM analysis in 2 out of 4 organs (13 weeks versus 104 weeks) with a fold change of >1.5 or <-1.5, III) an overall expression change of >1.5 or <-1.5 across 4 organs.

**Figure 7.** Quantitative real-time PCR of various microRNAs in 13, 104 and 130 week old livers (A), and NIH3T3 cells exposed to UVC (8 J/m2). Relative expression is shown (B).

**Figure 8.** Cross-hybridization controls for miR-16 en let-7a. The relative intensity of perfect matched capture probes and capture probes with 1 mismatch against miR-16 or let-7a are shown. Normalized to perfect matched capture probe. Note that one mismatch reduces hybridization intensity tremendously and decreases the likelihood that cross-hybridization accounts for observed microRNA expression regulation.

**Figure 9.** Heatmap of microRNAs that are significantly regulated (FDR < 5%, SAM analysis, minimal fold change of >1.5 or <-1.5) at least in one out of four 104 week old tissues compared to 13 week old tissues. Blue indicates down-regulation, red up-regulation. Grey boxes indicate that these microRNAs are not expressed above background levels in the specified organ.

**Figure 10.** Heatmap of microRNAs that are significantly regulated (FDR < 5%, SAM analysis, minimal fold change of >1.5 or <-1.5) in 130 week old
livers and the respective regulation of these microRNAs in 52 and 104 week livers. Blue indicates down-regulation, red up-regulation. Fold changes as seen in scale bars. Panel I) 14 commonly regulated microRNAs. Panel II) additional microRNAs regulated in 130 week old livers. Panel III) see panel II, but with other scale-bar. Note the progressive regulation for many microRNAs during aging.

**Figure 11.** MicroRNA expression during dietary restriction (DR). A. Distribution of the up- and down-regulation of all microRNAs in dietary restricted livers from C57BL/6 mice. Note that there is an even distribution between up- and down-regulated microRNAs, which indicates that the observed downregulation of 13 out of 14 microRNAs is not the result of an altered distribution or a general repression of microRNA expression. B. heatmap of microRNAs that are significantly regulated in dietary restricted livers (FDR<5% SAM analysis, fold change <-1.5 or >1.5) and their regulation in 104 and 130 week old livers from C57BL/6 mice. Blue indicates down regulation, red indicates up regulation. Let-7d-star, miR-200b, miR-122a, miR-130a and miR-30a-5p are significantly regulated in aged livers.

**Figure 12.** Heatmap of microRNAs that are significantly regulated (FDR<5%, SAM analysis, minimal fold change of >1.5 or <1.5) in UVC-treated NIH3T3 cells and/or human dermal fibroblasts (HDF) 4 hours after UVC treatment. Blue indicates down-regulation, red up-regulation. Panel I) UVresponsive microRNAs from 14 commonly regulated microRNAs. Panel II) additional microRNAs regulated after UV-C treatment. All microRNAs of panel II are also significantly regulated in 130 week old livers (Supplemental Figure 5). miR-23b and miR-24 are also regulated across all 2 year old tissues (Supplemental Figure 4). For linking UV-responsive microRNAs to gene expression arrays all microRNAs from panel I have been used and miR-23a, miR-23b and miR-422b.

**Figure 13.** Full length 3’UTRs of indicated genes (except IGF1: first 2.4 kb of 3’UTR) are cloned downstream Renilla luciferase and transiently co-
transfected with the indicated microRNA (expression mimicking oligo) in HEK293T cells. Empty pSICHECK II and non-targeting mimicking oligo were used as controls. 24 hours later cells were lysed and used for bioluminescence measurements. Panel I) control 3'UTR was co-transfected with indicated microRNA. None of these microRNAs regulate this control 3'UTR or Renilla luciferase itself. Panel II) 3'UTRs of indicated genes are co-transfected with either a control non-targeting mimicking oligo (grey bars), a microRNA that is not predicted to regulate the specified 3'UTR (blue bars) or the predicted microRNA (red bars).

Figure 14. Gene expression regulation in ERCC1 $^{8/}$ mutant livers. A. Panel I) The direction of gene expression regulation in gene expression arrays from 17 week old ERCC1 $^{8/}$ mutant livers (compared to age-matched littermate controls) was determined for significantly regulated genes from 130 week old livers that are predicted gene targets of 14 commonly regulated microRNAs. Only matching probe IDs were used. A fold change of $<-1.1$ or $>1.1$ is taken as cut-off. Panel II represents the direction of regulation of all probe IDs within the gene expression arrays from 17 week old ERCC1 $^{8/}$ livers. B. see A, only with a fold change cut-off of $-1.2$ or $>1.2$. Note that the level of expression changes is much more modest than seen after UV-C treatment or in 130 week old livers, which correlates with the level of expression of the 14 commonly regulated microRNAs in these situations. C. The expression level of predicted microRNA target genes is lower in 17 week ERCC1 $^{8/}$ livers compared to 130 week old livers or UV-C treated cells.

Figure 15. List of FOX03 target genes derived from Ingenuity Pathway Analysis.

Figure 16. MicroRNA-induced cell survival. The indicated microRNAs were ectopically up-regulated in NIH3T3 cells. 24 hours after transfection, cells were put at 0.5% serum and 24 hours later irradiated with UVC (12J/m2). 24 hours later, the number of living cells was counted. A. single microRNA up-regulation. Note that miR-16 and miR-29a reduce cell survival.
B. double microRNA up-regulation. MicroRNAs that induce cell survival are dominant over microRNAs that decrease cell survival. 

C. Western Blotting of NIH3T3 cells transfected with either a miR-21, miR-26a or miR-26b mimicking oligonucleotide or a control mimicking oligonucleotide in 2 concentrations. It was shown that miR-21 can regulate PTEN (Meng et al., 2007 Gastroenterology 133, 647), however we did not observe any regulation of PTEN by miR-21 in NIH3T3 cells. Note that PTEN is not among the miR-21 target genes (also not the non-conserved in mouse and human) in targetscan 5.1. In contrast, it was found that miR-26 regulates processes downstream of PTEN, that miR-26a and PTEN are needed for cell survival after UV-challenge, that PTEN down-regulation is required for cell survival after UV challenge and that it regulates lifespan-controlling pathways in the opposite direction as seen in longevity.

**Figure 17.** A. Relative expression levels of p21cip1, cullin 3 and NRF2 (NFE2L2) in UV-C treated cells, ERCC1<sup>−/−</sup>-livers and 130 week old livers in Affymetrix gene expression arrays compared to their own controls. B. list of NRF2 target genes.

**Figure 18.** Silencing of miR83 (C. elegans ortholog of miR29-a) results in marked survival of the worm. Hence, silencing of one or more of the miRNAs indicated herein are capable of prolonging lifespan, or prolonging cell lifecycle or improve anti-oxidant response.

**DETAILED DESCRIPTION**

As used herein the term "ageing genotype" refers to the specific expression level of the 14 miRNAs as described herein, wherein the expression of said miRNAs associated with the ageing genotype is upregulated during aging as compared to reference conditions. The various reference conditions and ageing associated conditions are indicated in Figure 5, wherein the profile of these 14 miRNAs shows upregulation in the case of 52, 104 and 130 weeks old mice as compared to normal 13 weeks old mice, wherein the profile of these
14 miRNAs shows downregulation when the mice are exposed to dietary restriction (a life prolonging treatment) compared to the aged mice, wherein the profile of these 14 miRNAs also shows the upregulation characteristic for normal ageing as mentioned before (52, 104 and 130 wk) in the case of mice having an accelerated ageing genotype (due to a mutation in the DNA repair gene ERCCI) at the age of 17 weeks, whereas at 4 weeks the characteristic profile is not expressed, wherein the profile of these 14 miRNAs shows no specific regulation in the case of 02, H202 or ionizing radiation challenge, and wherein the profile of these 14 miRNAs shows overall upregulation also in the case of UV challenge, which UV challenge is considered to indicate lasting DNA damage. Hence, the term "ageing genotype" refers to a genotype associated with ageing that is induced by DNA damage, e.g. UV induced or associated, age induced or associated, and/or accelerated ageing induced or associated DNA damage.

As used herein the term "nucleic acid" refers to multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymidine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). The term includes reference to polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymidine, inosine, 5- methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties. Other such modifications are well known to those of skill in the art. Thus, the term nucleic acid also encompasses nucleic acids with substitutions or modifications, such as in the bases and/or sugars.

The term "age-related disorder" refers to disorders associated with senescence. Representative age-related disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, diabetes, atherosclerosis,
osteoporosis, memory loss, arthritis, cardiovascular diseases, high blood pressure, stroke, aneurism, sarcopenia, age-related liver and kidney dysfunction, progeria, wrinkles/skin blemishes/liver spots, obesity, cancer, pain, urinary incontinence, locomotor dysfunction, sterility, sexual dysfunction, and dementia.

The term "inhibitory nucleic acid" refers to a nucleic acid specific for a target nucleic acid and inhibits the expression of the target nucleic acid. Representative inhibitory nucleic acids include, but are not limited to siRNA, miRNA, antisense RNA, DNA, or a combination thereof. Expression of the target nucleic acid can be inhibited at the transcriptional or translational level.

As used herein, the term "microRNA" refers to any type of interfering RNA, including but not limited to, endogenous microRNA and artificial microRNA. Endogenous microRNAs are small RNAs naturally present in the genome which are capable of modulating the productive utilization of mRNA. The term artificial microRNA includes any type of RNA sequence, other than endogenous microRNA, which is capable of modulating the productive utilization of mRNA.

"MicroRNA flanking sequence" as used herein refers to nucleotide sequences including microRNA processing elements. MicroRNA processing elements are the minimal nucleic acid sequences which contribute to the production of mature microRNA from precursor microRNA. Precursor miRNA termed pri-miRNAs are processed in the nucleus into about 70 nucleotide pre-miRNAs, which fold into imperfect stem-loop structures. The microRNA flanking sequences may be native microRNA flanking sequences or artificial microRNA flanking sequences. A native microRNA flanking sequence is a nucleotide sequence that is ordinarily associated in naturally existing systems with microRNA sequences, i.e., these sequences are found within the genomic sequences surrounding the minimal microRNA hairpin in vivo. Artificial microRNA flanking sequences are nucleotides sequences that are not found to
be flanking to microRNA sequences in naturally existing systems. The artificial microRNA flanking sequences may be flanking sequences found naturally in the context of other microRNA sequences. Alternatively they may be composed of minimal microRNA processing elements which are found within naturally occurring flanking sequences and inserted into other random nucleic acid sequences that do not naturally occur as flanking sequences or only partially occur as natural flanking sequences. The microRNA flanking sequences within the precursor microRNA molecule may flank one or both sides of the stem-loop structure encompassing the microRNA sequence. Preferred structures have flanking sequences on both ends of the stem-loop structure. The flanking sequences may be directly adjacent to one or both ends of the stem-loop structure or may be connected to the stem-loop structure through a linker, additional nucleotides or other molecules. As used herein a "stem-loop structure" refers to a nucleic acid having a secondary structure that includes a region of nucleotides which are known or predicted to form a double strand (stem portion) that is linked on one side by a region of predominantly single-stranded nucleotides (loop portion). The terms "hairpin" and "fold-back" structures are also used herein to refer to stem-loop structures. Such structures and terms are well known in the art. The actual primary sequence of nucleotides within the stem-loop structure is not critical as long as the secondary structure is present. As is known in the art, the secondary structure does not require exact base-pairing. Thus, the stem may include one or more base mismatches. Alternatively, the base-pairing may not include any mismatches.

Small RNA molecules are single stranded or double stranded RNA molecules generally less than 200 nucleotides in length. Such molecules are generally less than 100 nucleotides and usually vary from 10 to 100 nucleotides in length. In a preferred format, small RNA molecules have 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides. Small RNAs include microRNAs (miRNA) and small interfering RNAs
(siRNAs). MiRNAs are produced by the cleavage of short stem-loop precursors by Dicer-like enzymes; whereas, siRNAs are produced by the cleavage of long double-stranded RNA molecules. MiRNAs are single-stranded, whereas siRNAs are double-stranded.

The term "miRNA" includes reference to Pri-miRNAs. Pre-miRNAs and mature miRNAs as well as orthologs and paralogs thereof. As used herein, the term "paralogues" indicates separate occurrences of a gene (or other coding sequence) in one species. The separate occurrences have similar, albeit nonidentical, sequences, the degree of sequence similarity depending, in part, upon the evolutionary distance from the gene duplication event giving rise to the separate occurrences. The term "paralogs" hence refers to naturally occurring variants.

The term "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that is not toxic. Generally, there is no particular limitation in the length of siRNA as long as it does not show toxicity. "siRNAs" can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the double-stranded RNA portion of a final transcription product of siRNA to be expressed can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. The double-stranded RNA portions of siRNAs in which two RNA strands pair up are not limited to the completely paired ones, and may contain nonpairing portions due to mismatch (the corresponding nucleotides are not complementary), bulge (lacking in the corresponding complementary nucleotide on one strand), and the like. Nonpairing portions can be contained to the extent that they do not interfere with siRNA formation. The "bulge" used herein preferably comprise 1 to 2 nonpairing nucleotides, and the double-stranded RNA region of siRNAs in which two RNA strands pair up contains preferably 1 to 7, more preferably 1 to 5 bulges. In addition, the "mismatch" used herein is contained in the double-stranded RNA region of siRNAs in which two RNA strands pair up, preferably 1 to 7, more preferably 1 to 5, in number. In a preferable mismatch, one of the
nucleotides is guanine, and the other is uracil. Such a mismatch is due to a mutation from C to T, G to A, or mixtures thereof in DNA coding for sense RNA, but not particularly limited to them. Furthermore, in the present invention, the double-stranded RNA region of siRNAs in which two RNA strands pair up may contain both bulge and mismatched, which sum up to, preferably 1 to 7, more preferably 1 to 5 in number.

The terminal structure of siRNA may be either blunt or cohesive (overhanging) as long as siRNA can silence, reduce, or inhibit the target gene expression due to its RNAi effect. The cohesive (overhanging) end structure is not limited only to the 3’ overhang, and the 5’ overhanging structure may be included as long as it is capable of inducing the RNAi effect. In addition, the number of overhanging nucleotide is not limited to the already reported 2 or 3, but can be any numbers as long as the overhang is capable of inducing the RNAi effect. For example, the overhang consists of 1 to 8, preferably 2 to 4 nucleotides. Herein, the total length of siRNA having cohesive end structure is expressed as the sum of the length of the paired double-stranded portion and that of a pair comprising overhanging single-strands at both ends. For example, in the case of 19 bp double-stranded RNA portion with 4 nucleotide overhangs at both ends, the total length is expressed as 23 bp. Furthermore, since this overhanging sequence has low specificity to a target gene, it is not necessarily complementary (antisense) or identical (sense) to the target gene sequence. Furthermore, as long as siRNA is able to maintain its gene silencing effect on the target gene, siRNA may contain a low molecular weight RNA (which may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule), for example, in the overhanging portion at its one end.

In addition, the terminal structure of the “siRNA” is not necessarily the cut off structure at both ends as described above, and may have a stem-loop structure in which ends of one side of double-stranded RNA are connected by a linker RNA. The length of the double-stranded RNA region (stem-loop portion)
can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the length of the double-stranded RNA region that is a final transcription product of siRNAs to be expressed is, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long.

Furthermore, there is no particular limitation in the length of the linker as long as it has a length so as not to hinder the pairing of the stem portion. For example, for stable pairing of the stem portion and suppression of the recombination between DNAs coding for the portion, the linker portion may have a clover-leaf tRNA structure. Even though the linker has a length that hinders pairing of the stem portion, it is possible, for example, to construct the linker portion to include introns so that the introns are excised during processing of precursor RNA into mature RNA, thereby allowing pairing of the stem portion. In the case of a stem-loop siRNA, either end (head or tail) of RNA with no loop structure may have a low molecular weight RNA. As described above, this low molecular weight RNA may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule.

"Antisense RNA" is an RNA strand having a sequence complementary to a target gene mRNA, and thought to induce RNAi by binding to the target gene mRNA.

"Sense RNA" has a sequence complementary to the antisense RNA, and annealed to its complementary antisense RNA to form siRNA. These antisense and sense RNAs have been conventionally synthesized with an RNA synthesizer, here the present invention, these RNAs can be intracellularly expressed from DNAs coding for antisense and sense RNAs (antisense and sense code DNAs) respectively using the siRNA expression system.

Changes may be made to increase the activity of the miRNA, to increase its biological stability or half-life, and the like. All such modifications to the nucleotide sequences encoding such miRNA are encompassed.

As used herein nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common
ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology. Methods for determining sequence similarity percentages (e.g., BLASTN using default parameters) are generally available. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

The inventors of the present invention studied the role of miRNAs in the control of DNA damage repair and elaborated on the function of this novel class of RNA molecules in ageing and ageing-related disorders that may occur due to dysfunctional DNA damage repair mechanisms.

Research on understanding the processes of ageing and disorders that occur more frequently over time and that are related to processes such as loss of DNA damage repair, which is one of the drivers of ageing, is generally hampered by useful tools to check whether treatment is effective. This is in part due to the fact that many of these processes take a long time. Understandably, if a compound, or a combination of compounds or a certain treatment is applied to reduce, treat or prevent ageing, or the ageing-related disorder, it may take months, and even years to see a significant effect when compared to non-treated individuals or subjects. The inventors of the present invention have now provided a useful tool that can be applied in such research. By exploring the role of miRNAs in ageing in life mammals as well as in cell culture, they have found that certain miRNAs are strongly regulated over time. A set of miRNAs was identified that appeared significantly up-regulated in aged tissue, where on the other hand, when ageing was delayed by
experiments using caloric restriction, or dietary restriction, the same miRNAs were significantly down-regulated in expression. This now provides a tool for the researcher in the field of ageing to see whether a certain cell, tissue, organ or individual (animal or human) has aged, or in what stage of ageing such cell, tissue, organ or individual is. Moreover, to rapidly monitor an effect of a treatment, being the application of a compound or combination of compounds, or any other therapeutic application, one will now be able to check whether ageing, or the ageing-related disorder has progressed or has been influenced. In general, when a treatment slows down ageing, or downward influences the progress of the disease or the progress of ageing (which is likely due to an increase in the level of DNA damage), the expression level of the miRNAs of the present invention will be lower as compared to non-treated cell, tissues, organs or individuals. On the other hand, when no effect on the expression level is found, it is likely that the treatment has not influenced the ageing process or the progression of the (ageing-related) disorder.

By no means have the inventors claimed treating ageing or an ageing-related disorder. However, what is claimed is a tool, a method, a kit of parts that is useful in ageing research and in the research towards influencing the level of DNA damage control, and through this, influencing the progression of ageing and/or the ageing-related disorders. The tool that is provided relates to the expression level of a subset of miRNAs as disclosed herein. These miRNAs have an increased expression level while the organism ages, while the expression level is down-regulated when the ageing is slowed down.

Clearly, ageing is a complex phenomenon. Ageing generally comes with an almost endless list of potential disorders that occur more frequently as the organism progresses in age. The chance of getting cancer, neurodegenerative diseases (hearing loss, loss of sight, etc.), Alzheimer's disease, diabetes, osteoporosis and many other diseases and disorders increases over time. Besides that, numerous features occur that are not directly associated with disease, but certainly are related to ageing. Examples are skin wrinkling,
graying hair, or hair loss in general. The miRNAs disclosed herein and especially their expression profiles now provide a useful tool to monitor also such ageing-related features, their progress and potentially the reduction thereof.

As shown in the examples, the following miRNAs were identified as being significantly up-regulated in aged tissue: let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; miR-451. Hence the expression level of these miRNAs in combination in a cell may be used as a profile, which profile, when compared to young and/or healthy control cell, may be used to indicate the aging status of that cell. The miRNAs in aspects of the present invention are preferably selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193. Still more preferably, miRNAs in aspects of the present invention are preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e. Very highly preferred miRNAs are miR-26a, miRNA-29a and miRNA-29b.

A number of other miRNAs were also found to be up-regulated when cells were treated such that DNA damage increased (UV-C irradiation): miR-23b, miR-23a, miR-24, miR-27a, miR-27b, miR-44b. These miRNA may also be used in aspects of the present invention. The miRNAs discussed herein are all known in the art. However, their specific role in gene-expression control is largely unknown, although progress is being made in what genes may be under the control of what miRNAs, or what set of genes may be controlled by what miRNA or what set of miRNAs. For instance, a confirmed target of let-7 is the apoptosis effector caspase 3 (Tsang and Kwok. 2008), suggesting that let-7 regulates cell death induction upon DNA damage. miR-16 is involved in tumor suppression, by regulating various proto-oncogenes such as CDC25a
and BCL2 (Cimmino et al. 2005; Pothof et al. 2009). miR-21 has an increased expression in human cancer and regulates the PTEN tumor suppressor gene (Meng et al. 2007) as well as the CDC25a gene (Wang et al. 2009).

Here, a miRNA signature associated with ageing is disclosed. The 14 miRNAs comprised in this signature are specifically and progressively up-regulated in various organs/tissues from wt mice, and correspondingly earlier in progeroid ERCCI^ mice. Furthermore, expression was strikingly reversed in wt mice that underwent DR, a treatment that delays ageing. 20-week old C57BL6 male mice were subjected to dietary restriction (DR) (to 70% of the normal food intake) for 6 weeks and their liver miRNA expression profiles were then compared to their own (at 26 weeks) ad libitum fed controls. This revealed that upon DR the following miRNAs were down-regulated: let-7a; let-7c; miR-16; miR-30a-5p; miR-193; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; let-7f; and miR-30e. Importantly, the regulation of these miRNAs in repair-deficient ERCCI^ mice suggests a link with unrepaired DNA damage as at least one of the regulatory factors. Indeed, most miRNAs within this set can be regulated by persistent (but not transient) DNA damage in a direct, cell-autonomous manner. miRNA up-regulation during ageing and after UV treatment appears functionally relevant as many predicted miRNA target genes are concordantly regulated in 2.5 year old livers and by persistent DNA damage. Together, these observations demonstrate the signature miRNAs as a useful tool in the stochastic DNA damage model of ageing in which intrinsic and exogenous sources of cellular damage generate -among others- persistent lesions in DNA that accumulate in time thereby promoting age-related gene expression changes and inducing age-related phenotypes/pathologies.

As shown herein, more than 30% of all significantly regulated genes in aged 2.5 year old livers harbor a predicted binding site for one or more of the significantly expressed miRNAs of the present invention, which is much higher than expected on the basis of coincidence. This strongly supports the notion
that the miRNAs of the present invention are directly related to gene-expression control that is directly linked to the process of ageing and ageing-related features, and ageing-related disorders and/or pathologies.

Detection of miRNAs or silencing of miRNAs may occur through the use of nucleic acids that bind specifically to the miRNA. The term "specifically binding to" in the context of a nucleic acid as defined herein refers in particular to a nucleic acid that is capable of hybridizing under stringent conditions to the said miRNA. In a preferred embodiment, the said nucleic acid has a sequence that is the complement of the sequence of the miRNA as provided herein.

The miRNAs described herein were essentially detected in mice. However, the data are equally relevant for human subjects. The miRNAs and the pathways that they regulate are highly conserved in eukaryotic organisms.

Diagnostic use of the miRNAs comprises determining the expression level of the miRNAs in the test organ or test cell and comparing the expression level to that of control organ or cell. In the Examples below, the change in expression is given as the fold change relative to the control tissue in a 13 wk old (i.e. mature) mouse. Such mice are mature with respect to having reached reproductive age. Such references or controls may also be used in aspects of the present invention.

Methods for detection of a miRNA in a sample are known to the skilled person and include Northern blotting, the use of micro- en macroarrays, in situ hybridization, single molecule detection in liquid phase, massively parallel sequencing and quantitative polymerase chain reaction (Q-PCR). The RNA in a sample is preferably amplified prior to detection of miRNA sequences. Methods for amplification of RNA sequences are known in the art and include reverse-transcriptase polymerase chain reaction (RT-PCR) and Nucleic Acid Sequence Based Amplification (NASBA). A preferred amplification method comprises quantitative amplification of the RNA sequences by, for example, the use of SYBR GREEN (Roche, Basel,
Switzerland) or fluorescently labeled Taqman probes (Applied Biosystems, Foster City, USA).

Primers for reverse transcriptase-mediated cDNA synthesis may be provided by the provision of a shared sequence to all miRNA sequences such as, for example, a poly(A)-tail by ligation or through action of a Terminal Transferase, followed by annealing of an adapter-oligo(dT) primer. Further methods comprise the use of a stem-loop primer, and/or the use of a miRNA-specific primer. The quantitative amplification of the RNA sequences, preferably by real-time PCR, preferably comprises a universal primer and a miRNA-specific primer.

The primers used for detection, cDNA synthesis and/or amplification preferably comprise RNA nucleotides, DNA nucleotides or modified nucleotides such as Locked Nucleic Acid (LNA) nucleotides, Peptide Nucleic Acid (PNA) nucleotides, and/or 2'-0-alkyl modifications, 2'-fluoro modifications, 4'-thio modifications, a phosphorotioate linkage, a morpholino linkage, a phosphonocarboxylate linkage. In a preferred embodiment, the length of a primer, preferably a miRNA-specific primer, is identical to the length of the specific miRNA. In a further preferred embodiment, the length of the miRNA-specific primer is shorter than the length of the miRNA, for example 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, or 23 nucleotides, depending on the length of the specific miRNA. The sequence of a primer, preferably a miRNA-specific primer, preferably comprises one or two mismatches compared to the sequence of the miRNA or the adapter sequence that is added to the miRNA, more preferably is identical to the sequence of the miRNA.

Methods and means for detection of one or more miRNAs in a sample are preferably provided as a kit. Said kit preferably comprises a set of primers, preferably at least one specific set of primers, enzymes such as a RNA-dependent DNA polymerase and/or a DNA-dependent DNA polymerase,
and at least one buffer for performing the reaction or reactions. Said kit constituents may be provided as dried material, for example after lyophilisation, or as a liquid.

If the amount of the pri-miRNA and/or pre-miRNA and/or miRNA in a sample of a subject is essentially the same as the reference value, target mRNA expression is not changed. In this case, the subject is not diagnosed as exhibiting an ageing genotype.

Of course, other kinds of reference values than those of healthy subjects can be used. For instance, a reference value can be used that represents the amount of the pri-miRNA and/or pre-miRNA and/or miRNA in a subject, a tissue or a cell exhibits an ageing genotype. In this case, a sample with an amount of the pri-miRNA and/or pre-miRNA and/or miRNA which is essentially the same as said reference value indicates that said subject, tissue or cell exhibits an ageing genotype. A sample wherein said value is much smaller or larger indicates a young and/or healthy genotype.

The invention therefore provides a method for determining whether a subject, a tissue or a cell exhibits an ageing genotype, the method comprising:

- obtaining a sample from said subject;
- determining in said sample the amount of the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype herein, selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193; still more preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193;
- comparing said amount with a reference amount; and
- determining from said comparison whether or not the subject, a tissue or a cell exhibits an ageing genotype.

The amount of the pri-miRNA and/or pre-miRNA and/or miRNA in a sample of a subject is preferably established using a binding compound. At least part of a sample is preferably contacted with such binding compound (optionally after previous processing of the sample), where after unbound components are preferably washed away and bound compounds are preferably visualized and quantified. One embodiment thus provides a method according to the invention for determining whether a subject, a tissue or a cell exhibits an ageing genotype, the method further comprising contacting at least part of said sample with at least one compound capable of specifically binding the pri-miRNA and/or pre-miRNA and/or miRNA. Of course, if only a part of said sample is used, a part is used which contains microRNAs so that a suitable test is carried out.

The sample is preferably a blood or plasma or urine sample.

The invention furthermore provides a kit of parts for carrying out a method according to the present invention. Further provided is therefore a kit of parts comprising:

- at least one compound capable of specifically binding the pri-miRNA and/or pre-miRNA and/or miRNA indicated herein as associated with an ageing genotype; and

- instructions for use of said compound for determining whether a subject, a tissue or a cell exhibits an ageing genotype.

"Sample" is used in its broadest sense as containing nucleic acids. A sample may comprise a bodily fluid such as blood or urine; the soluble fraction of a cell preparation, or an aliquot of media in which cells were grown; a chromosome, an organelle, or membrane isolated or extracted from a cell; genomic DNA, RNA, or cDNA in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, buccal cells, skin, or hair; and the like.
A preferred sample for detection of a miRNA according to the invention is a body fluid selected from blood and urine. A most preferred sample is a blood sample. A blood sample may comprise a whole blood sample, or a sample that is obtained by centrifugation and/or filtration such as, for example, plasma, serum, platelets, red blood cell, white blood cells, as is known to the skilled person. A blood sample may be obtained by venepuncture, arteripuncture and/or capillary puncture such as, for example, a finger prick. The sample, preferably a blood sample, may be collected in a tube comprising an anticoagulant such as a heparin tube or an EDTA-tube, as is known to the skilled person.

In yet another embodiment, a method is provided for screening, detection and/or identification of candidate compounds capable of counteracting, treating, diminishing, delaying and/or preventing ageing. In this embodiment, candidate compounds are typically screened for their potential capabilities of decreasing the expression, amount and/or activity of a miRNA of which the upregulation is associated with the ageing genotype, or which have capabilities of counteracting the effects of the aberrant protein expression associated with the aberrant miRNA expression as indicated herein. If a candidate compound appears to have this property, it is capable of counteracting or preventing the ageing genotype. Further provided is therefore a method for determining whether a candidate compound is capable of counteracting, treating, diminishing, delaying and/or preventing the ageing genotype, the method comprising:

- contacting said candidate compound with a cell, preferably with a liver, kidney, spleen or lung cell, and

- determining whether said candidate compound is capable of counteracting in said cell the effects of protein expression associated with the upregulated miRNA expression as indicated herein.

In one preferred embodiment, a candidate compound is contacted with a cell according to the present invention wherein expression of the
miRNA (preferably which is upregulated in the ageing genotype) is silenced, so that the effects of candidate compounds upon the miRNA expression or activity is clearly visible. It is, however, also possible to use any cell or non-human animal currently known in the art in a screening method according to the invention. In one embodiment, a non-human animal or human cell is used.

Further provided is therefore a method for determining whether a candidate compound is capable of counteracting, treating, diminishing, delaying and/or preventing the aging genotype, the method comprising:

- contacting said candidate compound with a cell, and
- determining whether said candidate compound is capable of silencing a miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, or a functional part or derivative thereof, within said cell, or whether said compound is capable of altering the ageing genotype miRNA profile as disclosed herein such that the miRNA expression levels of said miRNAs are diminished and the cells miRNA profile moves towards a reference or control profile.

In a preferred embodiment, a method for diagnosing a subject as exhibiting the ageing genotype according to the invention comprises (a) determining the level of at least one miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193, still more preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e, or precursor microRNAs (pre-miRNA) or microRNA primary transcripts thereof and (b) diagnosing whether the subject exhibits the ageing genotype based on the level
of said at least one genetic markers determined in step (a). Very highly preferred miRNAs are miR-26a, miRNA-29a and miRNA-29b.

The sequences of the mature miRNAs are:

<table>
<thead>
<tr>
<th>microRNA</th>
<th>sequence mature microRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29a</td>
<td>tagcaccatctgaaatcggtta</td>
</tr>
<tr>
<td>miR-29b</td>
<td>tagcaccattgaaatcggttt</td>
</tr>
<tr>
<td>miR-26a</td>
<td>tcaagtaatccaggatagct</td>
</tr>
<tr>
<td>miR-26b</td>
<td>tcaagtaatccaggataggt</td>
</tr>
<tr>
<td>miR-21</td>
<td>tagctttacagctgatgttga</td>
</tr>
<tr>
<td>miR-16</td>
<td>tagcagcacgtaatatgggct</td>
</tr>
<tr>
<td>miR-22</td>
<td>aagctgccagttgaaactgt</td>
</tr>
<tr>
<td>miR-30a-5p</td>
<td>tgtaaacatcctgactggaag</td>
</tr>
<tr>
<td>miR-30e</td>
<td>tgtaaacatcctgactggaag</td>
</tr>
<tr>
<td>miR-451</td>
<td>aacccggtaccattactgagtt</td>
</tr>
<tr>
<td>miR-193</td>
<td>aactgcctacaatgtccagt</td>
</tr>
<tr>
<td>let-7a</td>
<td>tgaggtagtagttgtatagtt</td>
</tr>
<tr>
<td>let-7c</td>
<td>tgaggtagtagttgtatagtt</td>
</tr>
<tr>
<td>let-7f</td>
<td>tgaggtagtagattgtatagtt</td>
</tr>
</tbody>
</table>

In a further preferred embodiment, a method according to the invention comprises determining the level of at least two miRNAs selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193 still more preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e, or precursor microRNAs (pre-miRNA) or microRNA primary transcripts thereof, such as two miRNAs, three miRNAs, four miRNAs, five
miRNAs, six miRNAs, seven miRNAs, eight miRNAs, nine miRNAs, ten miRNAs, eleven miRNAs, twelve miRNAs, thirteen miRNAs or all miRNAs selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193 still more preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e, or precursor microRNAs (pre-miRNA) or microRNA primary transcripts thereof.

**Therapeutic use of the miRNAs of the present invention.**

The present invention relates to the diagnostic, prognostic and therapeutic use of miRNAs indicated herein. In methods of treatment according to the present invention, the subject is preferably a mammal, most preferably a human, and the miRNAs as indicated herein refer in preferred embodiments to the homo sapience (hsa) miRNAs.

Now that the invention has provided the insight that the hereinmentioned miRNAs are associated with an ageing genotype, it has become possible to diagnose, prevent and treat disorders associated with the ageing genotype. The association between the miRNAs described herein and the ageing genotype is through specific gene products which expression is prevented or regulated by the miRNAs. Counteracting the expression of certain of these specific gene products will result in a lower amount of these specific gene products, which are associated with the ageing genotype. This is particularly true for gene products whose expression is regulated by those miRNAs that are upregulated in the ageing genotype. According to the present invention, one or a combination of the miRNAs selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more
preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193, still more preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e are associated with ageing and the disorders associated therewith and counteracting the expression of these miRNAs can counteract the effects of the the ageing genotype.

One embodiment therefore provides a method for counteracting, treating, diminishing, delaying and/or preventing disorders associated with the ageing genotype, in a subject, the method comprising:

- decreasing the expression, amount and/or activity of an miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193. Still more preferably, miRNAs in aspects of the present invention are preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e or a functional part or derivative thereof, within said subject, and/or

- decreasing the interaction of the specific target mRNA with a miRNA selected from the group consisting of from selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193. Still more preferably, miRNAs in aspects of the present invention are preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e, or with a functional part or derivative thereof, within said subject.
In one embodiment, said subject is a subject exhibiting the ageing genotype as described herein, preferably characterized by the diagnostic profile representing the fingerprint of the ageing cell or tissue, including a premature ageing phenotype. In another embodiment, however, said subject is a subject not having the ageing genotype, hence, in an alternative embodiment, a method according to the invention is performed in order to generally prevent ageing, in an unaffected (young and/or healthy) subject.

Methods of the invention are suitable for therapeutic use for treating the effects associated with ageing genotype. In one preferred embodiment a method according to the invention is used in order to counteract, treat, diminish, delay and/or prevent the ageing genotype or disorders associated therewith. Further provided is therefore a method for treating and/or preventing disorders associated with ageing in a subject.

It is possible to decrease the expression, amount and/or activity of a miRNA upregulated in the ageing genotype as indicated herein, for instance by administering an antisense RNA (or other interfering nucleic acid as indicated herein) capable of silencing the miRNA. It is also possible to increase the expression, amount and/or activity of the mRNA that is the target of the miRNA, for instance by increasing the target mRNA gene expression.

In a preferred embodiment, the amount of the miRNA is decreased by administering a compound capable of silencing the pri-miRNA and/or the pre-miRNA and/or the miRNA, or a functional part or derivative thereof, to said subject. Administration of any of these compounds, or any combination of these compounds, results in silencing of the target miRNA and, thus, counteraction and/or prevention of the ageing genotype. The pri-miRNA is the primary transcript of the miRNA, having a length of several kilobases, which is obtained after transcription of the genomic miRNA sequence by RNA polymerase II. Pre-miRNA is the shorter, 70-100 nucleotide stem-loop structure which is obtained after processing of the pri-miRNA by the ribonuclease Drosha.
A functional part of the pri-miRNA or pre-miRNA or miRNA is defined herein as a nucleic acid sequence with a length of at least 7 nucleotides which is shorter than the pri-miRNA or pre-miRNA or (mature) miRNA, respectively, and which is capable of binding a target mRNA gene (the gene encoding the mRNA to which the miRNA binds) and/or target mRNA and counteracting expression of the protein from the target mRNA or the gene. Said functional part is preferably capable of binding the same 3’ UTR region of target mRNA which is capable of being bound by the miRNA. Said functional part preferably comprises a sequence of the seed region of the miRNA. A seed region of the miRNA is present in the 5’ region of the naturally occurring miRNA. Said seed region is particularly involved in target recognition. The sequence of the seed region of the miRNA can be found by the skilled artisan without difficulty or inventive skill by performing binding experiments between the miRNA and the mRNA. A functional part of the pri-miRNA or pre-miRNA or miRNA is thus preferably a nucleic acid sequence with a length of at least 7 nucleotides, comprising the seed region sequence. However, slight modifications of said seed region are allowed, as long as the capability of binding a target mRNA gene or the target mRNA is maintained. A functional part of the pri-miRNA or pre-miRNA or miRNA thus also comprises a nucleic acid sequence with a length of at least 7 nucleotides, comprising a sequence which has at least 72%, more preferably at least 80%, more preferably at least 86%, most preferably at least 90% sequence identity with the sequence of the seed region of the miRNAs as indicated herein.

A functional derivative of the pri-miRNA or pre-miRNA or miRNA is defined herein as a molecule which comprises a sequence which has 70% or more, but less than 100%, sequence identity with the pri-miRNA or pre-miRNA or miRNA and which has at least one same property in common irrespective of quantitative considerations. Said functional equivalent is capable of binding a target mRNA gene and/or target mRNA and counteracting expression of the protein encoded by the said gene, albeit not necessarily to the
same extent as the pri-miRNA or pre-miRNA or miRNA. Such functional equivalent for instance comprises:

- a polynucleotide wherein one or more nucleotides are added to the native sequence of the pri-miRNA or pre-miRNA or miRNA;

- a pri-miRNA or pre-miRNA or miRNA molecule wherein between 1-40% of the nucleotides are substituted by one or more other nucleotides, as long as the seed region sequence retains at least 72% sequence identity with the miRNA sequence as indicated in Figure 3; and/or

- a pri-miRNA or pre-miRNA or miRNA derivative wherein at least one nucleotide has been modified so that the resulting product has a non-naturally occurring nucleotide. Preferably, a functional equivalent of the pri-miRNA or pre-miRNA or miRNA has a nucleotide sequence having at least about 75% sequence identity with the pri-miRNA or pre-miRNA or miRNA, preferably at least about 80%, more preferably at least about 85%, more preferably at least about 90%, most preferably at least about 95%. The higher the sequence identity, the more closely said functional equivalent resembles the pri-miRNA or pre-miRNA or miRNA.

A preferred example of a functional equivalent of the pri-miRNA or pre-miRNA or miRNA is a vector comprising at least the seed region of the miRNA, or a vector comprising a sequence which has at least 72%, preferably at least 80%, more preferably at least 86%, most preferably at least 90% sequence identity with the seed region sequence.

The term "% sequence identity" is defined herein as the percentage of nucleotides in a nucleic acid sequence that is identical with the nucleotides in a nucleic acid sequence of interest, after aligning the sequences and optionally introducing gaps, if necessary, to achieve the maximum percent sequence identity. Methods and computer programs for alignments are well known in the art. As used herein, the terms "nucleic acid sequence" and "nucleotides" also encompass non-natural molecules based on and/or derived from nucleic acid sequences, such as for instance artificially modified nucleic acid
sequences, peptide nucleic acids, as well as nucleic acid sequences comprising at least one modified nucleotide and/or non-natural nucleotide such as for instance inosine.

Methods for introducing polynucleotides (such as anti-miRs) into a cell are known in the art. Methods for introducing nucleic acid for instance comprise calcium phosphate transfection, DEAE-Dextran, electroporation or liposome-mediated transfection. Alternatively, direct injection of the polynucleotide is employed. Preferably however, a nucleic acid sequence is introduced into a cell by a vector, preferably a viral vector. Said vector preferably comprises a retroviral, adenoviral, adeno-associated viral (AAV), or lentiviral vector. In one embodiment an AAV9 vector is used.

Various terms are known in the art which refer to introduction of nucleic acid into a cell by a vector. Examples of such terms are "transduction", "transfection" and "transformation". Techniques for generating a vector with a nucleic acid sequence and for introducing said vector into a cell are known in the art.

Preferably, the anti-sense RNA against the pri-miRNA or pre-miRNA or miRNA or a functional part or derivative thereof is used which is able to be introduced into a mammalian cell in vivo. Non-limiting examples of methods according to the invention are the coupling of said nucleic acid sequence to cell-penetrating peptides, to microcarriers or to nanocarriers, or the use of liposomes containing said nucleic acid sequence.

The above-mentioned compounds, or any combination thereof, are thus particularly suitable for the preparation of a medicament or prophylactic agent against disorders associated with the ageing genotype. Further provided is therefore a use of anti-pri-miRNA and/or anti-pre-miRNA and/or anti-miRNA as indicated herein, or a functional part or derivative thereof, or a use of a compound capable of inhibiting the expression, amount and/or activity of the miRNA in a cell, or a use of a compound capable of decreasing interaction of the target mRNA with the said miRNA, for the preparation of a medicament
or prophylactic agent against disorders associated with the ageing genotype. Said prophylactic agent is particularly suitable for subjects with a premature aging phenotype, as well as for subjects already suffering from ageing related disorders. However, said prophylactic agent is also suitable for generally preventing ageing, in unaffected subjects.

The above can also be accomplished by increasing or decreasing the amount or activity of the protein product encoded by the target mRNA of the miRNAs as identified herein. Administration of any of the above mentioned compounds, or any combination thereof, to a subject is particularly suitable for counteracting and/or preventing the effects of ageing or ageing related disorders, in said subject. In one embodiment, any of the above mentioned compounds, or any combination thereof, is administered to a subject who has been diagnosed with the aging genotype. In another embodiment, any of the above mentioned compounds, or any combination thereof, is administered to a subject with an increased risk of suffering from aging related disorders, for instance a subject who is already suffering from premature ageing. However, said compounds, or any combination thereof, are also suitable for generally preventing ageing, in unaffected subjects.

Methods for establishing the ageing status of a subject, tissue or cell, or methods for establishing and diminishing the risk of disorders associated with ageing becoming manifest, are presently unavailable. It has now been found that a method can be provided which includes measurement of the accumulation of (pri/pre) miRNA levels or the levels of the proteins encoded by the target mRNAs as identified herein. However, now that the present invention has disclosed the presence of a connection between a group of miRNAs and the ageing genotype, additional methods have become available. According to the invention, the presence or risk of suffering from ageing related disorders is established by determining whether the amount of the pri-miRNA and/or pre-miRNA and/or miRNAs associated with the ageing genotype as identified herein in a sample of a subject is above or below a
certain reference value. In one embodiment said reference value represents the amount of pri-miRNA and/or pre-miRNA and/or miRNA selected from the group consisting of selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, more preferably from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193. Still more preferably, miRNAs in aspects of the present invention are preferably selected from the group consisting of miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e in a healthy subject or healthy population, or a young adult not suffering from ageing related disorders. If the amount of the said pri-miRNA and/or pre-miRNA and/or miRNA in a sample of a subject is significantly above the reference value, the expression of the target mRNA is significantly counteracted. As a result, expression of the protein encoded by the target mRNA will be too low and the subject is suffering from, or at risk of suffering from, ageing related disorders. In such case, a therapy according to the present invention is recommended.

Therapeutic silencing of microRNA may be achieved by the use of LNA-mediated microRNA silencing such as een described by Lanford, et al. Science 327, 198 (2010) and Elmen et al., 2008. Nature 452, 896 (2008), the contents of which are incorporated by reference herein in their entirety. In short, such methods are based on treatment with an LNA-modified oligonucleotide that is complementary to the target miRNA. This treatment leads to long-lasting suppression of the miRNA, with no evidence of side effects in the treated animals, but with a concomitant derepression of target mRNAs with miRNA seed sites.

Essentially, therapeutic methods involving down-regulation of miRNAs may involve the administration to a subject in need thereof of a therapeutically effective dosage of an antisense RNA, for instance by injection into the body of an amount of about 5 mg/kg of body weight. Such experimental
results have been accomplished and demonstrated therapeutically effective results for mice and monkeys. A temporary knockdown of the target miRNA may be accomplished for a period of about 3 months, without side effects such as immune reactions, after which additional administration is optional.

Pharmaceutical Compositions and Therapeutic Uses

Pharmaceutical compositions can comprise polypeptides, polynucleotides or small molecules of the claimed invention, collectively called pharmaceutical compounds herein. The pharmaceutical compositions will comprise a therapeutically effective amount of either a biomarker protein, a polynucleotide or small molecule as described herein.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels. Therapeutic effects also include reduction in physical symptoms. The precise effective amount for a subject will depend upon the subject’s size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation and is within the judgment of the clinician.

For purposes of the present invention, an effective dose will be from about 0.01 mg/kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the polynucleotide or polypeptide compositions in the individual to which it is administered.

A pharmaceutical composition can also contain a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as a polypeptide, polynucleotide, and other therapeutic agents. The term refers to any
pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington’s Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier.

Delivery Methods

Once formulated, the pharmaceutical compositions of the invention can be (1) administered directly to the subject; (2) delivered ex vivo, to cells derived from the subject; or (3) delivered in vitro for expression of recombinant proteins.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The
compositions can also be administered into the nervous system. Other modes of administration include topical, oral, suppositories, and transdermal applications, needles, and particle guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Methods for the \textit{ex vivo} delivery and re-implantation of transformed cells into a subject are known in the art and described in e.g., International Publication No. WO 93/14778. Examples of cells useful in \textit{ex vivo} applications include, for example, stem cells, particularly hematopoietic, lymph cells, macrophages, dendritic cells, or tumor cells.

Generally, delivery of nucleic acids for both \textit{ex vivo} and \textit{in vitro} applications can be accomplished by, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

Various methods are used to administer the therapeutic composition directly to a specific site in the body. Receptor-mediated targeted delivery of therapeutic compositions containing an antisense polynucleotide, subgenomic polynucleotides, or antibodies to specific tissues is also used. Receptor-mediated DNA delivery techniques are described in, for example, Findeis \textit{et al.}, Trends in Biotechnol. (1993) 11:202-205; Wu \textit{et al}, J. Biol. Chem. (1994) 269:542-46.

Pharmaceutical compositions containing polynucleotides are preferably administered in a range of about 100 ng to about 200 mg of polynucleotides for local administration in a gene therapy protocol. Concentration ranges of about 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of polynucleotides can also be used during a gene therapy protocol. Factors such as method of action and efficacy of transformation and expression are considerations which will affect the dosage required for ultimate efficacy of the
polynucleotides. Where greater expression is desired over a larger area of tissue, larger amounts of polynucleotides or the same amounts re-administered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions of, for example, a nerve ending or synaps, may be required to affect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect. A more complete description of gene therapy vectors, especially retroviral vectors, is contained in WO 98/00542, which is expressly incorporated herein.

The invention will now be illustrated by way of the following, non limiting Examples.

EXAMPLES

Example 1.

**miRNA expression changes in aged mouse tissues**

To study the role and expression levels of miRNAs in the course of normal ageing, 4 tissues (liver, lung, kidney, spleen) were profiled from adult (13 weeks), middle-aged (1 year) and aged (2 years) wild type (C57BL6) male mice from an ageing cohort of which at 2 years of age the majority of wild type mice were still alive. As apparent from a complete autopsy at macroscopic and microscopic level (data not shown), mice used for this study did not have any obvious pathology, which could influence results and were therefore considered as normally aged and healthy animals.

miRNA expression profiles were generated using locked nucleic acid based miRNA arrays containing capture probes against 328 known mouse miRNAs. To perform this miRNA expression analysis, total RNA was isolated from freshly frozen tissue (or cell cultures, see below) using Trizol (Invitrogen). miRNA arrays were performed and analyzed as described by Pothof et al. (2009). Affymetrix gene expression arrays (430, V2.0) were performed and
analyzed as described (Schumacher et al. 2008; Garinis et al. 2009). Statistical analyses of arrays, generating heat maps and unsupervised hierarchical clustering were performed with MeV4.5.1 (Saeed et al. 2003). Quantitative RT-PCR of genes and miRNAs was performed as described (Garinis et al. 2009; Pothof et al. 2009). C57BL/6 mice from the ageing cohort and dietary restriction (see below) were housed and analyzed as described (Wijnhoven et al. 2005). ERCCI\(^{\text{A}}\) mice (see below) were housed and analyzed as described by Niedernhofer et al. (2006).

First, the expression data of 4 tissues and 3 ages were classified by unsupervised hierarchical clustering (Fig 1A), which shows a primary clustering on tissue type and a secondary clustering on age: in all tissues 13 week and 1 year samples are more similar than 2 year old tissues indicating that age-related miRNA expression changes are prominent in each tissue.

A search for systematic miRNA expression changes across these 4 tissues revealed a set of 14 commonly regulated miRNAs in these organs (Fig IB; figure 6). All these miRNAs (miR-21, miR-193, miR-30a-5p, miR-22, miR-16, miR-26a, miR-29a, let-7c, let-7a, let-7f, miR-30e, miR-26b, miR-29b, and miR-451) were up-regulated over time. The strongest effect was seen in liver, while up-regulation in the lung was relatively modest. Notably, numerous paralogs (equivalents) of the same miRNA families that differed only one or a few nucleotides were identified and tested. Notably, control capture probes on the array with only 1 mismatch in its sequence, had a more than 95% reduction in hybridization signal, indicating that cross-reactivity is unlikely (data not shown).

A search for systematic microRNA expression changes across these 4 organs revealed a set of 14 commonly regulated microRNAs (i.e. ~4% of total) (Figure IB; Figure 6, qRT-PCR controls Figure 7), all were up-regulated during aging, the strongest in liver, least pronounced in lung. Moreover, various paralogs of the same microRNA families differing only one or a few nucleotides were identified, unlikely to be due to cross-hybridization (Figure
We identified additional microRNAs regulated across all 4 tissues, but to a lesser extent as seen in Figure 1B (did not reach stringent criteria from Figure 6), and tissue-specific microRNAs (Figure 9).

Since up-regulation of these commonly regulated miRNAs appeared progressive and most pronounced in liver, the expression levels in 2.5 years old C57BL6 male mouse livers, which belong to the 15% longest living mice from the cohort (all pathology free), were determined. It was found that the same 14 miRNAs were significantly up-regulated (False Discovery Rate less than 5%) and appeared even more pronounced than in 2 year old livers (Fig 1C) confirming the progressive nature of this change during ageing.

**Example 2.**

**Relationship between miRNA expression and ageing**

To further explore the relationship between the expression levels of the 14 miRNAs that were found in the previous example and ageing, the effect of a somewhat delayed and accelerated ageing on the observed miRNA profile was examined.

It is generally known and accepted in the field of ageing research that dietary restriction (DR) (also referred to as caloric restriction: CR) increases lifespan and delays the onset of age-related pathology in numerous organisms, including mice and primates (Fontana et al. Science 328; 321-). 20-week old C57BL6 male mice were subjected to DR (to 70% of the normal food intake) for 6 weeks and their liver miRNA expression profiles were then compared to their own (at 26 weeks) *ad libitum* fed controls. This revealed that upon DR 13 out of the identified 14 miRNAs were down-regulated (Fig ID).

A defect in specific DNA repair pathways such as Nucleotide Excision Repair (NER) in combination with Transcription-Coupled Repair (TCR) or cross-link repair causes the appearance of many ageing features (Niedernhofer et al. 2006). For instance, defects in the NER/cross-link repair gene Ercc1 cause segmental ageing in liver, kidney and hematopoietic, skeletal and
neuronal systems, which limits the life span to ~25 weeks and gives gene expression profiles that resemble natural ageing (Schumacher 2008). To elaborate on this, the expression levels of the 14 miRNAs detected as disclosed herein were checked in mice that have a mutant Erccl gene (Erccl<sup>+/−</sup>-

While at 4 weeks none of these 14 miRNAs were differently expressed as compared to age-matched littermate controls, 17 weeks old Erccl<sup>+/−</sup> mice exhibited a liver miRNA expression shift similar to that of 2 year old wt mice (Fig IE; fold change is shown compared to age-matched wt littermates). It was concluded that these changes reflect the ageing status and that the accumulation of DNA damage that occurs in these mutant mice contributes to this dramatic expression level change. It is in fact in accordance with the idea that lifelong accumulation of persistent DNA damage (in wt organisms) contributes to the ageing process. Because stochastic accumulation of DNA damage likely occurs in all cells in all tissues during ageing, DNA damage or cellular stress may directly affect age-regulated miRNA expression, for which the set of 14 miRNAs seems representative.

Example 3.

**Effect of DNA damage on miRNA expression levels**

Various types of DNA lesions can occur each with their specific characteristics. To investigate the effect of DNA damage on miRNA expression profiles, we induced several of such lesions by different means and checked for expression.

First, the induction of oxidative lesions was investigated by using hydrogen peroxide on NIH3T3 cells as well as by using a shift in oxygen concentration to 20% on primary Mouse Embryonic Fibroblasts (MEFs). For this, NIH3T3 cells were grown in DMEM (Gibco-BRL); human dermal fibroblasts (HDFs) and HEK293T were grown in a 1:1 mixture of DMEM and Ham's F10 medium (Gibco-BRL). Medium was supplemented with 10% FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml). NIH3T3 cells at ~70-
80% confluency were treated with H2O2 (tert-Butyl hydroperoxide solution; Sigma) by mixing with the cell culture’s medium in 15 ml tubes and added to the cell cultures. Primary MEFs were cultured at 3% oxygen and shifted to 20% oxygen. LY294002 (in DMSO; Biomol Int.) was added to the medium 16 hrs before protein isolation at a final concentration of 10 μM. Insulin (Sigma) was added at a final concentration of 5 pg/ml.

Oxidative stress lesions are generally quickly repaired (Dizdaroglu et al. 2005). Whereas these treatments caused up-regulation of the DNA damage responsive, p53-controlled p21cip1 product (data not shown), they failed to trigger the ageing-related miRNA expression patterns that were reported herein (Fig IF: NIH3T3 cells: 4 hrs after H2O2 (100 μM) treatment. Fold change is shown compared to mock-treated cells. MEFs: 8 hrs after shifting from 3% O2 to 20% O2. Fold change is shown compared to primary MEFs grown at 3% O2), indicating that at least these types of oxidative damage are unlikely to be involved in the change of expression of this set of miRNAs.

Second, cyclobutane pyrimidine dimers were checked. These lesions are induced by UV light and are an example of persistent DNA damage, which remains in most parts of the genome for days or longer. Recently, it was shown that these types of transcription-blocking lesions may serve as a surrogate for persistent DNA damage in ageing (Garinis et al. 2009). Moreover, NER/TCR deficiency, which is linked with premature ageing, causes extreme sensitivity to these types of DNA damage. Dividing cultures of NIH3T3 cells and Human Dermal Fibroblasts (HDFs) were irradiated with UV-C (Philips TUV germicidal lamp) at ~70-80% confluence. UV treatment of HDFs induced significant up-regulation of 8 of the 14 miRNAs reported herein, namely miR-16; miR-30a-5p; miR-21; miR-22; miR-26a; miR-26b; miR-29a; and miR-29b, whereas up-regulation was seen with 12 out of the 14 miRNAs when NIH3T3 cells were used, namely let-7a; let-7c; miR-16; miR-30a-5p; miR-193; miR-21; miR-22; miR-26b; miR-29a; miR-29b; let-7f; and miR-30e (Fig 1G: 4 hrs after UV-C irradiation (8J/m²); fold changes compared to mock-treated cells). It was
found that additional miRNAs, namely miR-23b, miR-23a, miR-24, miR-27a, miR-27b, miR-44b, were also significantly regulated after UV treatment, which were also up-regulated upon ageing in 130 week old livers and to a lesser extent across 4 tissues (Fig 12). Thus, persistent DNA damage in cultured cells and unrepaired endogenous DNA damage in Ercc1^- livers causes the regulation of various miRNAs, which phenomenon is also observed in aged organs/tissues, strengthening the parallel between the alterations induced by persistent DNA damage and ageing.

Example 4.

**Link between miRNAs and genes involved in ageing**

The differences in miRNA expression between young adult and old organs likely contribute to changes in gene expression. Since miRNAs predominantly induce mRNA degradation that can be monitored in microarray analysis (Guo, H. et al., 2010. Nature, 466, 835-840), it was reasoned that at least part of significantly down-regulated genes in ageing observed in genome-wide gene expression profiling could be the result of miRNA up-regulation and *vice versa*. For this Targetscan 5.1 for miRNA-gene interaction predictions was used (Grimson et al. 2007. Mol Cell 27, 91) and the focus was on predicted miRNA-gene interactions that are conserved in mammals. The significantly regulated genes were derived from 2.5 years old livers and three miRNA sets for the miRNA-gene interaction predictions were selected:

- **Set 1**: all regulated miRNAs in 2.5 year old livers (Fig 1C);
- **Set 2**: the 14 commonly regulated miRNAs across 4 tissues (Fig 1B);
- **Set 3**: all overlapping miRNAs regulated in 2.5 years old livers and after UV damage (Fig 1G; Figure 12)

As controls five random gene sets were used to calculate enrichment, which is expected when the regulatory actions of these miRNAs is reflected in
the 2.5 years old liver gene expression profile. About 1/3 of all significantly regulated genes from 2.5 years old livers appeared to have at least one predicted miRNA binding site for one of the significant regulated miRNAs from Set 1 (Fig 2A, panel I), which is a significant >2.3-fold overrepresentation.

Subsequently, this analysis was repeated for Set 2 and Set 3. Then, a 2.8 and 2.5 fold enrichment, respectively was observed (Fig 2A, panel I), which is more than all miRNAs together. This indicates the importance of these miRNAs in ageing-related gene-expression control. Swindell (BMC Genomics 10, 585, 2009) published an elaborate meta-analysis from gene expression profiles of 22 aged mouse tissues (which also entailed the gene expression profiles of the 2.5 year old livers used here) that included the 300 most down-regulated genes across these 22 tissues. It was investigated whether the set of 14 miRNAs of the present invention would apply to more genes, which could be reflected in these gene expression data. Indeed, even 43% of all these genes appeared to have at least one predicted, conserved miRNA binding site for one of the 14 signature miRNAs of the present invention, which is almost a 5 fold enrichment (Fig 2A, panel II). This strongly strengthens the significance of this relationship.

To further examine the reliability of the predicted miRNA-gene interactions, the 3'-UTRs of 16 age-regulated genes were cloned downstream of a Renilla luciferase reporter gene and the regulation of those 3'-UTRs after ectopic up-regulation of the predicted regulatory miRNA was monitored. Functional evidence was found for 15 out of 16 miRNA-gene interactions (data not shown).

Since UV damage induces similar miRNA expression changes as observed in ageing, it was expected that the 'ageing genes' that are likely regulated by DNA damage inducible miRNAs (Set 3, Fig 2A; Figure 12) are also regulated after UV irradiation. Gene expression profiles of UV-treated NIH3T3 cells were generated (these were the same samples as used for
miRNA profiling). It was observed that the majority of these genes was also
down-regulated in UV-irradiated cells with a large part having an FDR of less
than 5% (Fig 2B, panel I). This is an enormous enrichment (p=1.0e-9, Fisher-
exact test) when compared to all genes in the array (Fig 2B, panel III). Also
most of the predicted gene targets from UV-inducible miRNAs in the 300 most
down-regulated genes across 22 tissues were down-regulated, many with an
FDR of less than 5% (significant enrichment: p=0.000011, Fisher Exact test)
(Fig 2B, panel II). Although miRNA expression changes were less pronounced
in 17 weeks old Erccl^- livers, which correlates with the observed intensity of
gene expression down-regulation, it was still observed that many genes were
down-regulated as expected, again representing a significant enrichment
(p=4.9e-13, Fisher Exact test) (Figure 14).

Material and methods.

Animals, cell culture and treatments.
C57BL/6 mice from the aging cohort and dietary restriction were housed and
analyzed as described(i). ERCC1^- mice were housed and analyzed as
described (Niedernhofer et al., 2006 Nature 444, 1038). Mouse embryonic
fibroblasts (MEFs) and NIH3T3 cells were grown in Dulbecco's modified
Eagle's medium (DMEM; Gibco-BRL), human dermal fibroblasts (HDFs) and
HEK293T in a 1:1 mixture of DMEM and Ham's F10 medium (Gibco-BRL).
Medium was supplemented with 10% fetal calf serum and penicillin (100 U/ml)
/ streptomycin (100 pg/ml). Dividing cultures of NIH3T3 cells and HDFs were
irradiated with UV-C (Philips TUV germicidal lamp) at ~70-80% confluency.
NIH3T3 cells at ~70-80% confluency were treated with H2O2 (Sigma) by
mixing with the cell culture's medium in 15 ml tubes and added to the cell
cultures. Primary MEFs were cultured at 3% oxygen and shifted to 20%
oxygen. LY294002 (in DMSO; Biomol Int.) was added to the medium 16 hours
before protein isolation at a final concentration of 10µM. Insulin (Sigma) was added at a final concentration of 5 pg/ml.

MicroRNA and gene expression analysis.

Total RNA was isolated from freshly frozen tissue or cell cultures using Trizol (Invitrogen). MicroRNA arrays were performed and analyzed as described (Pothof et al., 2009 EMBO J 28, 2090). Affymetrix gene expression arrays (430a, V2.0) were performed and analyzed as described (Schumacher et al., 2008 PLoS Genet 4, e1000161; Garinis et al., 2009. Nat Cell Biol 11, 604).

Statistical analyses of arrays, generating heatmaps and unsupervised hierarchical clustering were performed with MeV4.5.1 (Saeed et al., 2003 Biotechniques 34, 374). Data is available through the public repository ArrayExpress at accession codes: EMEXP-839, E-MEXP-1503 and E-MEXP-2978. Overrepresented pathway analysis was performed using Ingenuity and DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). Quantitative RT-PCR of genes and microRNAs was performed as described (Pothof et al., 2009 EMBO J 28, 2090; Garinis et al., 2009. Nat Cell Biol 11, 604). For linking microRNA and gene expression data, we compared significant up regulated microRNAs with significant down regulated genes and vice versa. We used Targetscan 5.1 (Lewis et al., 2005 Cell 120, 15; Grimson et al., 2007 Mol Cell 27, 91; Friedman et al., 2009 Genome Res 19, 92) for microRNA target predictions. We only used those predictions that were broadly conserved among vertebrates. Poorly conserved microRNA target genes were discarded. We only used those significantly regulated genes that were also present in the Targetscan 5.1 database. Genes that were not present in this database were discarded. As a control we used random gene sets with a similar number of genes. We discarded all genes that had a low hybridization signal in the micro-arrays, indicating nonexpressed genes, or were not present in the Targetscan 5.1 database. Pathway analysis on these random gene sets using DAVID (http://david.abcc.ncifcrf.gov/summary.jsp) did not result in any significant
regulated pathways confirming the random selection of genes (data not shown). For the determination of overlap between putative microRNA-regulated genes in aging and after UV irradiation, the direction of gene expression regulation in gene expression arrays from UV-C treated NIH3T3 cells was determined. Only significant Affymetrix probe IDs from 130 week old livers were matched. For the 300 most down-regulated genes across 22 tissues in mouse aging, which are predicted target genes of overlapping microRNAs between the 14 commonly regulated microRNAs and UV-C treated NIH3T3 cells, specific Affymetrix probe IDs were not specified. Therefore, either one specific probe ID for a gene was used when significantly regulated (SAM analysis; FDR<5%) or the average fold change from all probe IDs together for the same gene was used.

**Transfection.**

NIH3T3 cells were transfected with Lullaby reagent (Oz Biosciences). MicroRNA expression mimicking oligonucleotides (miRIDIAN mimics) were obtained from Dharmacon, on-target plus siRNAs from Dharmacon (3 to 4 different siRNAs per gene were used) and microRNA antisense microRNAs (miRCURY LNA microRNA inhibitors) were obtained from Exiqon. SiRNAs and miRIDIAN mimics were transfected at 50 nM final concentrations. The miRCURY LNA microRNA inhibitors against miR-26a and miR-26b were co-transfected at 25 nM final concentrations each. Double-transfections with siRNAs and miRIDIAN mimics or miRCURY LNA microRNA inhibitors was done at the above mentioned concentrations. In our experiments we did not observe differences between control miRCURY LNA microRNA inhibitors (Exiqon), control siRNAs (Dharmacon) or control miRIDIAN mimics (Dharmacon) with final concentrations of 50 or 100 nM (data not shown). Control oligonucleotides were transfected at 50 nM final concentrations or 100 nM when the experiment included double transfections with siRNAs and miRIDIAN mimics or miRCURY LNA microRNA inhibitors. 48 hours after
transfection, experiments were performed. The 6xDBE construct (Furuyama et al. 2000 Biochem J 349, 629), GFP-NRF2 vector (Furukawa and Xiong 2005 Mol Cell Biol 25, 162) and pSICHECK II vector (Promega) were transfected with Lullaby reagent (Oz Biosciences).

Protein isolation and western blots.

Following treatment, cells were washed twice with ice-cold PBS and directly lysed into Laemmli sample buffer. Extracts were boiled for 5 minutes at 95°C and subjected to SDS-PAGE analysis, according to standard lab techniques. Primary antibodies used: PKB T308p, PKB S473p and PTEN (Cell Signalling), Foxo3a T32p (Upstate), P21cip1 (BD Pharmingen), α-Tubulin (DM1A; Calbiochem), GAPDH (Millipore) and PKB (home-made; described previously (Van Weeren et al. 1998 J Biol Chem 273, 13150).

Luciferase assays.

3'UTRs were cloned downstream a Renilla luciferase in a pSICHECK II vector (Promega). HEK293T cells were co-transfected using Lullaby (Oz Biosciences) with the indicated miRIDIAN mimic (Dharmacon). Luciferase activity was measured 24 hours later by Dual Glow luciferase kit (Promega). For real-time monitoring of 6xDBE driven luciferase, the 6xDBE-luciferase construct and miRCURY LNA microRNA inhibitors were co-transfected in NIH3T3 cells using Lullaby (Oz Biosciences). 24 hours later HEPES (25 nM final concentration) and 0.1 mM luciferin (Sigma) were added to the medium. Again 24 hours later, cells were irradiated with UV-C and bioluminescence was monitored for 16 hours (75 s measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C.
Claims

1. A method for determining whether a subject, a tissue or a cell exhibits an ageing genotype, the method comprising:
   - providing a sample from said subject;
   - determining in said sample the amount of the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451;
   - comparing said amount with a reference amount; and
   - determining from said comparison whether or not the subject, a tissue or a cell exhibits an ageing genotype.

2. Method for determining an ageing stage of a subject, a tissue or a cell, the method comprising:
   - providing a sample from said subject or tissue or a cell;
   - determining in said sample the amount of the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an advanced ageing stage selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451;
   - comparing said amount with a reference amount; and
   - determining from said comparison whether or not in the subject, tissue or cell said miRNAs are upregulated relative to said reference.

3. Method according to claim 1 or 2, wherein said amount of the pri-miRNA and/or pre-miRNA and/or miRNAs is determined in the form of a miRNA profile for miRNAs let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-
26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, preferably miRNAs let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; and miR-193, still more preferably miRNAs miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; and miR-30e.

4. A method according to claim 1, 2 or 3 wherein the reference amount is an internal standard.

5. A kit of parts adapted for use in a method according to any one of claims 1 to 4, said kit comprising:

- at least one compound capable of specifically binding the pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451; and

- instructions for use of said compound for determining whether a subject, a tissue or a cell exhibits an ageing genotype.

6. A method for determining whether a candidate compound is capable of counteracting, treating, diminishing, delaying and/or preventing an ageing genotype or a disorder associated with ageing, the method comprising:

- contacting said candidate compound with a cell, tissue or subject, preferably with a liver, kidney, spleen or lung cell, and

- determining whether said candidate compound is capable of silencing one, preferably all, miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, or determining whether said candidate compound is capable of preventing or counteracting the decrease in
protein expression associated with the upregulation of said miRNAs observed in an ageing genotype.

7. Method according to claim 6 further comprising the steps of:
   - assaying the survivability of the cell, tissue or subject contacted with the candidate compound or the severity of the disorder;
   - comparing the survivability or the severity of the disorder of the cell, tissue or subject contacted with the test compound to a control cell, tissue or subject; and
   - selecting the compound that prolongs the survivability or ameliorates (the effects of) the disorder of the cell, tissue or subject contacted with the test compound compared to survivability of the control cell, tissue or subject.

8. A method for counteracting, treating, diminishing, delaying and/or preventing disorders associated with the ageing genotype, in a subject, the method comprising:
   - decreasing the expression, amount and/or activity of a miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, within said subject, and/or
   - decreasing the interaction of the specific target mRNA with a miRNA selected from the group consisting of let-7c; let-7a; let-7f; miR-16; miR-21; miR-22; miR-26a; miR-26b; miR-29a; miR-29b; miR-30a-5p; miR-30e; miR-193; and miR-451, within said subject.

9. The method of claim 8 wherein the age-related disorder is selected from the group consisting of Alzheimer's, Parkinson's, dementia, and other forms of age-related neurodegeneration such as retinal degeneration and deafness, diabetes, metabolic syndrome, atherosclerosis, cardiovascular diseases, arthritis, osteoporosis.
10. The method of claim 9 wherein the subject is a human.
Figure 2

A

I

II

% predicted mRNA target genes

1. all genes
2. common upregulated and downregulated
3. UV-responsive microRNA


B

I

II

III

no change (-1.2<FC<1.2)
up regulation
down regulation FDR<5%

C

all liver aging genes

p-value (log)

oxidative phosphorylation
ubiquitin-mediated proteolysis
ubiquitin-mediated proteolysis
ubiquitin-mediated proteolysis
citrulline cycle (TCA cycle)
insulin signaling pathway
adipocyte differentiation
pyruvate metabolism
PKC pathway
androgen and estrogen metabolism
mitochondria to cytosol acetyl-glycine-shuttling
biphasicity of unesterified fatty acids
VEGF signaling pathway
TGF-beta signaling pathway
farnesoid X receptor (CAR)
Klf4 signaling pathway
AKT/mTOR signaling pathway
SREBP pathway of lipid synthesis
TGF/Smad RAS-CAMP signaling
Spred regulation of TGF-α/FGFR-3
proteasome
EGF signaling pathway
ERK signaling pathway
PTEN signaling pathway
JNK signaling pathway

D

liver aging microRNA target genes (predicted)

p-value (log)

insulin signaling pathway
ubiquitin mediated proteolysis
focal adhesion
adipocyte differentiation
AKT/mTOR signaling pathway
PTEN signaling pathway
ErbB signaling pathway
apoptosis
JNK signaling pathway
NOS3 signaling pathway
PKC pathway
taurine metabolism
citrulline cycle (TCA cycle)
ethe lipid metabolism
TGF-beta signaling pathway
eIF-4 pathway
light chain
VEGF signaling pathway
regulation of actin cytoskeleton

E

UV-responsive microRNA target genes (predicted)

p-value (log)

ubiquitin mediated proteolysis
insulin signaling pathway
focal adhesion
PTEN signaling pathway
PKC pathway
JNK signaling pathway
AKT/mTOR signaling pathway
ErbB signaling pathway
apoptosis
eIF-4 pathway
GnRH signaling pathway
Figure 4

A

Mt2
Hmgb1
Hmgb2
Htt
Mrox1
Hoxa1
Akr1b3
Elovl4
Peg3
Akr1b8
Getm1
Acor5
Cht1
Asc1
Gstn2
Gstn1
Dlvb5
Pdg
Mod1
Eph31
Adh7
Akr7a5
Mpxt1
Gstp1
Sod2
Cst
Txl1
Txd1

fold change

-2 1 2

C

GFP-NRF2

I

nucleus (+)

II

% GFP-NRF2 positive cells

0% 20% 40% 60% 80% 100%

III

% GFP-NRF2 positive cells

0% 20% 40% 60% 80% 100%

control

miR-26a

Pten

pten

miR-26a

miR-26a

miR-26a

miR-26a

pten

pten

pten

4h after UVC (8 J/m²)

B

DMSO

LY294002

control

miR-26a

Foxo2

T28-P

P21

PTEN

GAPDH

D

E

F

persistent transcription
blocking DNA lesions
(e.g. by UV)

miR-26a

PTEN

P21

NRF2

cell preservation

protection against future insults
from ROS and other damaging
compounds
Figure 5
Figure 6

I) Group 1
\[ \Delta 13w \text{ (n=3) and } \Delta 104w \text{ (n=3) liver} \]
\[ \Delta 13w \text{ (n=3) and } \Delta 104w \text{ (n=3) lung} \]
\[ \Delta 13w \text{ (n=3) and } \Delta 104w \text{ (n=3) kidney} \]
\[ \Delta 13w \text{ (n=3) and } \Delta 104w \text{ (n=3) spleen} \]

Two-Class Paired SAM

Select microRNAs:
FDR < 5%
Fold change > 1.5 or < -1.5

II) 13w (n=3) vs 104w (n=3) liver
13w (n=3) vs 104w (n=3) lung
13w (n=3) vs 104w (n=3) kidney
13w (n=3) vs 104w (n=3) spleen

\[ \rightarrow \text{ two-class unpaired SAM} \]

Select microRNAs:
FDR < 5%
Fold change > 1.5 or < -1.5

III) Overall expression change of > 1.5 or < -1.5 across
4 tissues and in 3 out of 4 at least > 1.5 or < -1.5.

MicroRNAs were selected as being commonly regulated between 13 week old and 104 week old tissues, when A) positive in I), B) positive in 2 out of 4 organs in II), and C) positive in III).
Figure 7

A

B
Figure 8

miR-16

relative intensity

let-7a

relative intensity

liver 13w, liver 104w, kidney 13w, kidney 104w, spleen 13w, spleen 104w, lung 13w, lung 104w

- perfect match
- 1 mismatch
Figure 9

[Diagram showing expression levels of various miRNAs in different tissues.]
Figure 10

III

fold change

-2.0 0 2.0

-3.5 1 3.5

miR-122a
miR-126
let-7e
miR-24
miR-422b
miR-194
miR-23a
miR-27b
miR-200b
miR-130a
miR-29c
miR-326
miR-346
miR-129-3p
miR-350
miR-93
miR-197
miR-299
miR-433

miR-16
miR-21
let-7c
let-7a
miR-29b
miR-30a-5p
miR-22
miR-193
miR-26a
miR-29a
miR-26b
let-7f
miR-30e
miR-451

-3.5 1 3.5

fold change
Figure 11

A

- fold change < -1.5
- -1.5 < fold change < -1.2
- -1.2 < fold change < 1.0
- 1.0 < fold change < 1.2
- 1.2 < fold change < 1.5
- fold change > 1.5

B

DR  104w 130w

miR-200b
miR-181b
let-7d-star
miR-122a
miR-30a-5p
miR-130a
miR-134
miR-293

fold change
Figure 12

NIH3T3 (4h)  
HDF (4h)

I

miR-16  
miR-26a  
miR-193  
miR-21  
miR-22  
miR-30a-5p  
miR-29a  
miR-29b  
miR-26b  
let-7a  
let-7f

II

miR-23b  
miR-23a  
miR-24  
miR-27a  
miR-27b  
miR-422b

fold change
**Figure 13**

The figure shows relative luminescence for various microRNAs and 3'UTRs. The microRNAs include control, let-7a, miR-16, miR-21, miR-22, miR-26a, miR-29a, and miR-30a-5. The 3'UTRs include CNOT2, CDC34, E1F4E, RNF138, CDC37L1, SPRY2, FUSIP1, LNX2, PRKAG2, IGF1, IGF1, COL5A3, DNMT3B, COL11A1, ANKRD17, TAO1, and SMURF2.
Figure 14

A

I

II

- no change (-1.1<FC<1.1)
- up regulation
- down regulation
- down regulation FDR<5%

B

I

II

- no change (-1.2<FC<1.2)
- up regulation
- down regulation
- down regulation FDR<5%

C

fold change predicted microRNA target genes

fold change

0.0 0.2 0.4 0.6 0.8 1.0

liver 180Pw UV/RadiF ERCC1 IV-1 (Fw)
Figure 15

A

ACOT1 ALDH3A1 APEX1 AR BBC3 BCL2L11 BNIP3 BNIP3L CAD CAT CCNB1 CCND1 CCND2 CCNE2 CCNG2 CDH1 CDK4 CDKN1A CDKN1B CPT1A CYR61 DDIT3 EXT1 FASLG FBXO32 FOXM1 GADD45A GPX1 GRB14 GSTM1 GTF2I HBP1 IARS IMPDH2 JAK1 LARS LCN2 MAX MKI67 MT1 MTHFD2 MXD1 MXD3 MXD4 MXI1 NOS3 PPARG1A PPM1D RBL2 RCAN1 SEPP1 SESN1 SLC1A4 SLC40A1 SLC7A1 SMAD4 SOD1 SOD2 TNFSF10 UBE2C UCP2
Figure 16
Figure 17

A

B

ABCC2 ABCC3 ABCC4 ABCC5 ADH7 AKR1B3 AKR1B7 AKR1B8 AKR1C12 AKR1C13 AKR1C14 AKR1C18 AKR1C19 AKR1C20 AKR1C21 AKR1C6 AKR7A5 ALDH3A1 BLVRB CBR1 CBR3 CYP2A12 CYP2A4 CYP2B13 CYP2B9 CYP2C39 CYP4A10 CYP4A14 EPHX1 FMO3 FTH1 FTL1 FTL2 G6PD2 GCLC GCLM GGT1 GPX2 GPX3 GSR GSTA1 GSTA2 GSTA3 GSTA4 GSTM1 GSTM2 GSTM3 GSTM4 GSTM5 GSTM6 GSTM7 GSTP1 GSTT1 GSTT2 GSTT3 GSTT4 H6PD HMOX1 ME1 MGST2 MGST3 MT1 MT2 MT3 MT4 NQO1 PGD PRDX1 PRDX6 PTGR1 SLC1A4 SLC7A11 SOD3 SULT3A1 TXN1 TXNRD1 TXNRD3 UCHL1 UCHL3 UCHL4 UCHL5 UGT2B35
Figure 18
### A. CLASSIFICATION OF SUBJECT MATTER

**INV.** C12Q1/68 G01N33/50

**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)**

C1Q  G01N

**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 practical, search terms used)

EPO-Internal, BIOSIS

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

* Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document 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.
* "Z" document member of the same patent family

**Date of the actual completion of the international search** 1 September 2011

**Date of mailing of the international search report** 06/01/2012

**Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016**

**Authorized officer** Schmitt-Humbert, C
**INTERNATIONAL SEARCH REPORT**

**DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
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<tbody>
<tr>
<td>A</td>
<td>HACKL MATTHIAS ET AL: &quot;mi R-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging&quot;, AGING CELL, vol. 9, no. 2, April 1 (2010-04), pages 291-296, XP002658047, abstract figure 1</td>
<td>1-10</td>
</tr>
<tr>
<td>A</td>
<td>NISHINO JINSUKE ET AL: &quot;Hmga2 Promotes Neural Stem Cell Self-Renewal in Young but Not Old Mice by Reducing pl6(Ink4a) and pl9(Arf) Expression&quot;, CELL, vol. 135, no. 2, October 2008 (2008-10), pages 227-239, XP002658049, ISSN: 0092-8674 figure 7</td>
<td>1-10</td>
</tr>
<tr>
<td>X</td>
<td>WO 2010/120969 A1 (UNIV TEXAS [US]; OLSOER I; R0019 EVA VAN [US]; FROST ROBERT [US]) 21 October 2010 (2010-10-21) claims 1, 2, 4, 18, 19, 30, 31</td>
<td>5-10</td>
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Form PCT/SA210 (continuation of second sheet) (April 2005)
## DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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</tr>
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</table>

Form PCT/ISA/210 (continuation of second sheet) (April 2005)
### 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.**:  
     - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### 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:

"see additional sheet"

1.  
   - Checkmark:  
     - As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.  
   - Checkmark:  
     - As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3.  
   - Checkmark:  
     - 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.  
   - Checkmark:  
     - 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.:

   I-1O(partial ly)

#### Remark on Protest

- Checkmark:  
  - The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

- Checkmark:  
  - 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.

- Checkmark:  
  - No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: I-10 (partially)

Method for determining whether a subject, a tissue or a cell exhibits an ageing genotype comprising determining the amount of the let-7c pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype; method for determining an ageing stage in a subject, a tissue or a cell comprising determining the amount of the let-7c pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an advanced ageing stage; a kit of parts for use in said methods comprising at least one compound capable of specifically binding the let-7c pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and/or an increased expression and/or delaying and/or preventing the decrease in protein expression associated with the upregulation of said miRNAs; and a method for counteracting, treating, diminishing, delaying and/or preventing disorders associated with the ageing genotype comprising decreasing the expression, amount and/or activity of the let-7c miRNA within the subject, and/or decreasing the interaction of the specific target miRNA with the let-7c miRNA within said subject.

2-14. claims: I-10 (partially)

Method for determining whether a subject, a tissue or a cell exhibits an ageing genotype comprising determining the amount of the X pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype; method for determining an ageing stage in a subject, a tissue or a cell comprising determining the amount of the X pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an advanced ageing stage; a kit of parts for use in said methods comprising at least one compound capable of specifically binding the X pri-miRNA and/or pre-miRNA and/or miRNA indicated as associated with an ageing genotype and/or delaying and/or preventing disorders associated with the ageing genotype comprising determining whether a candidate compound is capable of counteracting, treating, diminishing, delaying and/or preventing an ageing genotype or a disorder associated with an ageing genotype and/or an increased expression and/or delay in the expression of the let-7c miRNA and/or the let-7c miRNA associated with the ageing subject.
protein expression associated with the upregulation of said miRNA; and a method for counteracting, treating, diminishing, delaying and/or preventing disorders associated with the aging genotype comprising decreasing the expression, amount and/or activity of the X miRNA within the subject, and/or decreasing the interaction of the specific target mRNA with the X miRNA within said subject;

wherein in invention 2, X is let-7a; in invention 3, X is let-7f; in invention 4, X is miR-16; in invention 5, X is miR-21; in invention 6, X is miR-22; in invention 7, X is miR-26a; in invention 8, X is miR-26b; in invention 9, X is miR-29a; in invention 10, X is miR-29b; in invention 11, X is miR-30a-5p; in invention 12, X is miR-3Qe; in invention 12, X is miR-193 and in invention 14, X is miR-451.
<table>
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