Chapter 1

MicroRNA-663 induction upon oxidative stress in cultured human fibroblasts depends on the chronological age of the donor

M.E.C. Waaijer*, M.Wieser*, R. Grillari-Voglauer, D. van Heemst, J. Grillari**, A.B. Maier**
* Both authors contributed equally

Biogerontology. 2014 Jun;15(3):269-78

Abstract

MicroRNAs, regulators of messenger RNA translation, have been observed to influence many physiological processes, amongst them the process of aging. Higher levels of microRNA-663 (miR-663) have previously been observed in human dermal fibroblasts subject to both replicative and stress-induced senescence compared to early passage cells. Also, higher levels of miR-663 have been found in memory T-cells and in human fibroblasts derived from older donors compared to younger donors. In previous studies we observed that dermal fibroblasts from donors of different chronological and biological age respond differentially to oxidative stress measured by markers of cellular senescence and apoptosis. In the present study we set out to study the association between miR-663 levels and chronological and biological age. Therefore we tested in a total of 92 human dermal fibroblast strains whether the levels of miR-663 in non-stressed and stressed conditions (fibroblasts were treated with 0.6μM rotenone in stressed conditions) were different in young, middle aged and old donors and whether they were different in middle aged donors dependent on their biological age, as indicated by the propensity for familial longevity. In non-stressed conditions the level of miR-663 did not differ between donors of different age categories and was not dependent on biological age. Levels of miR-663 did not differ dependent on biological age in stressed conditions either. However, for different age categories the level of miR-663 in stressed conditions did differ: the level of miR-663 was higher at higher age categories. Also, the ratio of miR-663 induction upon stress was significantly higher in donors from older age categories. In conclusion, we present evidence for an association of miR-663 upon stress and chronological age.

Introduction

Although widely studied, molecular mechanisms contributing to the process of aging in humans have not yet been fully uncovered. Interestingly, some individuals appear to age slower and healthier than others. This slower rate of aging was previously studied in a unique cohort of Caucasian offspring of long lived families. These offspring were observed to have a lower mortality rate, beneficial glucose and lipid metabolism and preservation of insulin sensitivity when compared with their partners as age and environmentally matched controls [1-3]. Furthermore, the *in vitro* stress response of dermal fibroblasts of these offspring was observed to mimic that of chronologically younger donors (i.e. fewer senescent cells) while the stress response of partners mimicked that of chronologically older donors (i.e. more senescent cells) [4]. These in vitro results are in line with recent findings showing that a higher number of p16INK4a positive cells in human skin is associated with higher biological age ex situ [5]. The importance of senescence in the aging process was previously observed *in vivo* as well, as clearance of p16INK4a positive cells was shown to delay age-related pathologies in mice [6].

MicroRNAs (miRNAs), a class of non-coding RNAs, are important regulators of messenger RNA translation [7]. Thereby, single miRNAs can regulate up to hundreds of mRNA targets and are therefore considered to act similar to transcription factors, modulating multiple physiological processes, amongst others the aging process [8;9]. The level of microRNA-663 (miR-663) was previously found to be higher in replicative senescence [10-12] and in stress-induced senescence [11] in human fibroblasts *in vitro,* as well as in memory T-cells [12]. Furthermore, a higher level of miR-663 in human foreskin fibroblasts from elderly versus young healthy donors was observed [12].

In the present study we investigated the level of miR-663 in human dermal fibroblasts in both non-stressed and stressed conditions, as well as the ratio thereof. We tested if these levels differ between donors of different chronological age categories and between middle aged subjects of a different biological age, namely offspring of nonagenarian siblings (with a propensity for familial longevity) and their middle aged partners.

Methods

Study design

The current study included three age categories consisting of young (n=8), middle aged (N=76) and old donors (N=8) derived from the Leiden 85-plus Study or the Leiden Longevity Study (LLS).

The Leiden 85-plus Study is a prospective population-based study in which all inhabitants aged 85 years of the city of Leiden (The Netherlands) were invited to take part [13]. A biobank was established from fibroblasts cultivated from skin biopsies from 68 of the 275 surviving 90-year-old participants [14] from December 2003 to May 2004. A biobank of fibroblasts from biopsies of 27 young donors (18-25 years) was established from August to November 2006.

In the LLS genetic factors contributing to familial longevity are studied. Middle aged Caucasian offspring from nonagenarian siblings (not related to the subjects of the Leiden 85-plus Study) were included together with their partners as age and environmentally matched controls. There were no selection criteria on health or demographic characteristics. From November 2006 to May 2008, a biobank was established from fibroblasts cultivated from skin biopsies from 150 offspring-partner couples.

The Medical Ethical Committee of the Leiden University Medical Center approved both studies and written informed consent was obtained from all participants.

Characteristics of the donors

Demographic characteristics were available for each donor. Information on medical history was obtained from the participants' treating physicians. Total number of cardiovascular diseases included cases of cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus.

Culture conditions and experimental set-up

Fibroblast strains were isolated from three (Leiden 85-plus Study) and four (LLS) mm biopsies of the sun unexposed medial side of the upper arm and cultured under predefined, highly standardized conditions as published earlier [14]. Fibroblasts were grown in D-MEM:F-12 (1:1) medium supplemented with 10% fetal calf serum (Bodinco, Alkmaar, the Netherlands, batch no. 162229), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM glutamax I, and antibiotics (100 Units/mL penicillin, 100 µg/mL streptomycin, and 0.25–2.5 µg/mL amphotericin B), all obtained from Gibco, Breda, the Netherlands unless stated otherwise. Fibroblasts were incubated at 37°C with 5% CO_2 and 100% humidity. Trypsin (Sigma, St Louis, MO, USA) was used to split fibroblasts using a 1:4 ratio each time they reached 80-100% confluence. Further experimental procedures have been published earlier as well [15]. In short, on day 0, passage 11 fibroblasts were thawed from frozen stocks and on days 4, 7 and 11, fibroblasts

were further passaged in order to multiply fibroblasts. The experiments were started on day 18. Fibroblast strains were seeded at 2300 and 3900 cells/cm^2 for non-stressed and rotenone-stressed cultures respectively. Fibroblast strains were seeded in batches of eight strains per condition. To stress fibroblast strains, medium was supplemented with 0.6 μM rotenone (Sigma, St Louis, MO, USA), known to induce an increase in the intracellular production of ROS at the mitochondrial level [16]. This particular concentration of rotenone was observed to give an stress-induced increase of SAβ-gal and the low percentage of apoptosis, as shown in previously published work [15]. After 72 hours fibroblasts were frozen in pellets from 92 randomly chosen donors (8 young and 8 old donors from the Leiden 85-plus Study and 38 offspring of the LLS together with 38 partners thereof) for RNA extraction.

RNA extraction

Total RNA was extracted by classical phenol–chloroform extraction [17]. In brief, cells were homogenized in 0.5 mL Tri-Reagent by vortexing for 15 seconds, incubated at room temperature for 5 minutes and vortexed again for 15 seconds. 100 μl chloroform (Emsure, Merck KGsA, 64271 Darmstadt, Germany) was added to the samples, which were then vortexed for 15 seconds and incubated at room temperature for 3 minutes. The samples were then centrifuged at 12000xg for 15 minutes at 4°C. After centrifugation the upper aqueous phase was transferred to a RNase-free tube. 1 μl of glycogen (Ambion, 5 mg/ml) and 250 μl of 100% isopropanol were added to this aqueous phase, followed by vortexing and 10 minutes incubation at room temperature. Afterwards the samples were centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was discarded and the RNA pellets were washed with 500 μl 75% ethanol. The samples were centrifuged at 7600xg for 5 minutes at 4°C. After discarding the ethanol the RNA pellets were air-dried for 10 minutes. The RNA was resuspended in 15 μl RNase-free water and dissolved by incubation for 10 minutes at 57°C. To improve the purity of the RNA and decrease possible residing phenol the samples were precipitated again by adding 1.5 μl of natrium acetate (3 M, pH 5.2), 1 μl of glycogen (Ambion, 5 mg/ml) and 18 μl of 100% isopropanol and incubated at -20°C overnight. The samples were then centrifuged at 9300xg for 15 minutes at 4°C and the supernatant removed. The pellets were washed with 500 μl 75% cold ethanol and centrifuged at 7600xg for 5 minutes at 4°C. The ethanol was discarded and the pellets were air-dried for 10 minutes. The pellets were then resuspended with 15 μl RNase-free water and incubated for 10 minutes at 57°C. The RNA concentration was quantified by using NanoDrop (ThermoScientific, Wilmington, USA).

qPCR

cDNA was synthesized from 100 ng of total RNA using the NCode™ VILO™ miRNA cDNA Synthesis Kit (Life technologies, Carlsbad, CA 92008, USA) and was diluted 1:5 with RNAse-free water. qPCR was performed using Sensimix SYBR® Hi-Rox Mastermix

(Bioline) and the Universal qPCR Primer (10 μM) of the NCode™ VILO™miRNA cDNA Synthesis Kit according to the manufacturer's instructions. Forward primers (1 pmol/μl) used were 5'-AGGCGGGGCGCCGCGGGAC for hsa-miR-663 and 5'-CAGGGTCGGGCCTGGTTAGTA for 5S rRNA (serving as a reference gene). The qPCR reactions were performed on a Rotor-Gene Q (Qiagen) thermocycler.

Statistics

Levels of miR-663 in fibroblasts in both non-stressed and stressed conditions were measured by qPCR and normalized to 5S rRNA, which has been used in various studies as a housekeeping gene in various settings and across various species [18-20], by subtracting the mean miR-663 cycling threshold (Ct) of 4 replicates with the mean 5S Ct of 4 replicates. These ΔCt's are hereafter named miR-663 levels in non-stressed and stressed conditions. To calculate the ratio of miR-663 induction upon stress the ΔΔCt method was used. With this ratio the miR-663 level in stressed conditions was related to the miR-663 level in non-stressed conditions. Data are expressed as log2 fold change for this ratio of miR-663 induction upon stress. Donors with a log2 fold change value being 3 standard deviations below or above the mean were excluded in all analyses (N=2, middle aged donors).

Table 1. Characteristics of the donors

	Leiden 85-plus Study		Leiden Longevity Study	
	Young (N=8)	Old (N=8)	Offspring (N=38)	Partners (N=38)
Demographic data				
Female, no.(%)	6 (75.0)	5 (62.5)	19 (50.0)	19 (50.0)
Age, years, mean (SD)	22.3 (1.0)	90.2 (0.5)	63.5 (7.1)	63.4 (7.7)
Anthropometric data, mean (SD)				
Body mass index, kg/m²	22.5 (2.0)	25.5 (3.7)	26.8 (4.7)[a]	25.8 (3.4)[b]
Co-morbidities, no. (%)				
Myocardial infarction	0 (0.0)	2 (25.0)	0 (0.0)[c]	0 (0.0)[a]
Cerebrovascular accident	0 (0.0)	1 (12.5)	1 (2.8)[a]	2 (5.6)[a]
Hypertension	0 (0.0)	3 (37.5)	9 (25.0)[a]	8 (22.2)[a]
Diabetes mellitus	0 (0.0)	2 (25.0)	2 (5.7)[c]	5 (14.3)[c]
Malignancies	0 (0.0)	1 (12.5)	1 (2.9)[b]	2 (5.9)[d]
Chronic obstructive pulmonary disease	0 (0.0)	1 (12.5)	1 (2.8)[a]	2 (5.7)[c]
Rheumatoid arthritis	0 (0.0)	3 (37.5)	0 (0.0)[a]	0 (0.0)[a]
Intoxications, no. (%)				
Smoking, current	0 (0.0)	1 (12.5)	6 (16.7)[a]	4 (11.1)[a]

SD: standard deviation, no.: number
a: N=36, b:N=37, c: N=35, d: N=34

Differences in levels of miR-663 in non-stressed and stressed conditions and the ratio thereof between young and old donors, and offspring and partners were analyzed with the use of linear mixed models. A linear mixed model differs from a standard regression model in the ability to take intra-individual repeated measurements into account. Further adjustments included potential random batch effects, gender, chronological age (the last in offspring and partner comparison only) and the number of cardiovascular diseases. Since hypertension was previously linked to miR-663 expression [21], the association between miR-663 and chronological age categories was studied as well separately for donors with and donors without cardiovascular diseases. All analyses were performed using SPSS editor software.

Table 2. Levels of microRNA-663 and ratio of induction upon stress dependent on chronological age categories

	Young (N=8)	Middle-aged (N=74)	Old (N=8)	P for trend
Levels of miR-663				
ΔCt non-stressed				
Model 1, estimated mean (SE)	-9.77 (0.35)	-9.65 (0.23)	-9.75 (0.35)	0.95
Model 2, estimated mean (SE)	-9.76 (0.37)	-9.69 (0.23)	-9.83 (0.36)	0.87
ΔCt stressed				
Model 1, estimated mean (SE)	-9.56 (0.35)	-9.53 (0.22)	-8.82 (0.35)	0.04
Model 2, estimated mean (SE)	-9.65 (0.36)	-9.56 (0.22)	-8.78 (0.36)	0.02
Ratio of induction upon stress				
log2 fold change stressed to non-stressed				
Model 1, estimated mean (SE)	0.06 (0.30)	0.27 (0.10)	0.76 (0.30)	0.09
Model 2, estimated mean (SE)	-0.03 (0.30)	0.27 (0.09)	0.92 (0.30)	0.04

SE: standard error, ΔCt: delta cycle threshold. Calculations were made with the ΔΔCt method, in which Ct values of microRNA-663 were normalised to those of a housekeeper gene. Higher ΔCt values indicate higher microRNA-663 expression. The log2 fold change was calculated as miR-663 expression levels in stressed to those in non-stressed conditions. Number of cardiovascular diseases include cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus. Donors with a datapoint 3 standard deviations below or above the mean were excluded from this analysis. The young donors are aged 21 to 24 years (mean 22 years), the middle-aged donors 44 to 73 years (mean 64 years) and the old donors 90 to 91 years (mean 90 years).
Model 1: adjusted for batch and repeated measurements
Model 2: adjusted for batch, repeated measurements, gender and number of cardiovascular diseases

Results

The present study included human dermal fibroblast strains from 92 donors, consisting of 8 young donors (mean age 22.3 years), 76 middle-aged donors (mean age 63.5 years) of whom

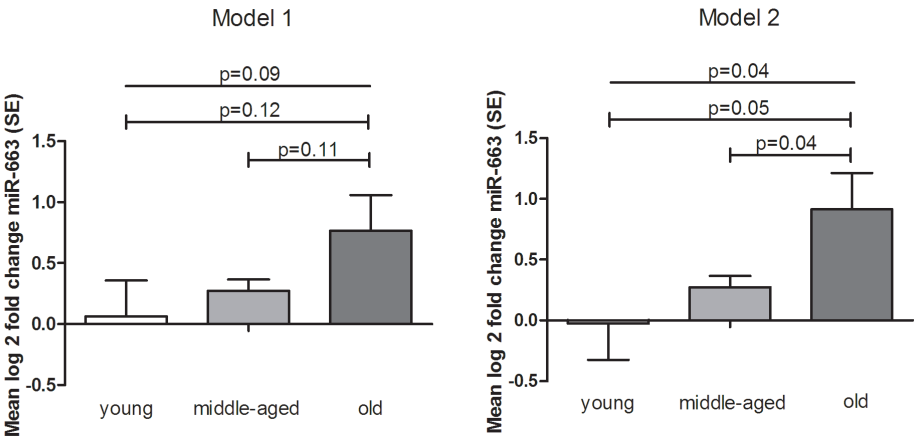

Figure 1. Ratio of microRNA-663 induction upon stress dependent on chronological age categories
SE: standard error, miR-663: microRNA-663. The log2 fold change was calculated with ΔΔCt method as miR-663 expression levels in stressed to those in non-stressed conditions. Number of cardiovascular diseases include cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus. The middle-aged group consists of both offspring and partners. Donors with a datapoint 3 standard deviations below or above the mean were excluded from this analysis. The young donors are aged 21 to 24 years (mean 22 years), the middle-aged donors 44 to 73 years (mean 64 years) and the old donors 90 to 91 years (mean 90 years).
Model 1: adjusted for batch and repeated measurements
Model 2: adjusted for batch, repeated measurements, gender and number of cardiovascular diseases

38 offspring and 38 partners (mean ages 63.5 and 63.4 years, respectively) and 8 old donors (mean age 90.2 years). The characteristics of donors are summarized in Table 1.

Table 2 shows the levels of miR-663 dependent on chronological age categories. The mean level of miR-663 normalized to 5S RNA in non-stressed conditions did not differ between the three chronological age categories, also after adjustment for gender and cardiovascular diseases. The mean level of miR-663 in stressed conditions was dependent on chronological age categories, showing significantly higher mean levels at higher ages. After adjustment for gender and cardiovascular diseases this association remained statistically significant.
The ratio of miR-663 induction upon stress, expressed as the ratio between miR-663 levels in stressed and non-stressed conditions from each individual, was also higher in higher chronological age categories. This association was significant after adjustment for gender and cardiovascular diseases. The ratio of miR-663 induction upon stress dependent on chronological age categories is visualized in Figure 1.
To disentangle the potential influence of cardiovascular disease on the association between miR-663 induction upon stress and chronological age categories we repeated the analysis

Table 3. Ratio of microRNA-663 induction upon stress dependent on chronological age categories, stratified on cardiovascular diseases

	Young	Middle-aged	Old	P-value
Subjects without CVD, N=57				
ΔCt non-stressed, estimated mean (SE)	-9.86 (0.37)	-9.83 (0.22)	-10.44 (0.58)	0.50
ΔCt stressed, estimated mean (SE)	-9.65 (0.38)	-9.46 (0.23)	-9.01 (0.61)	0.31
Log2 fold change stressed to non-stressed, estimated mean (SE)	0.06 (0.31)	0.39 (0.12)	1.50 (0.58)	0.05
Subjects with one or more CVD, N=26				
ΔCt non-stressed, estimated mean (SE)	n/a	-9.71 (0.29)	-9.60 (0.43)	0.80
ΔCt stressed, estimated mean (SE)	n/a	-9.79 (0.26)	-8.81 (0.41)	0.02
Log2 fold change stressed to non-stressed, estimated mean (SE)	n/a	0.01 (0.17)	0.43 (0.34)	0.27

SE: standard error, CVD: cardiovascular diseases, ΔCt: delta cycle threshold. Calculations were made with the ΔΔCt method, in which Ct values of microRNA-663 were normalised to those of a housekeeper gene. Higher ΔCt values indicate higher microRNA-663 expression. The log2 fold change was calculated as miR-663 expression levels in stressed to those in non-stressed conditions. Number of cardiovascular diseases includes cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus. Only 5 donors had more than one cardiovascular disease. Donors with a datapoint 3 standard deviations below or above the mean were excluded from this analysis. Adjusted for batch effects, repeated measurements and gender. The young donors are aged 21 to 24 years (mean 22 years), the middle-aged donors 44 to 73 years (mean 64 years) and the old donors 90 to 91 years (mean 90 years).

Table 4. Levels of microRNA-663 and ratio of induction upon stress in offspring of nonagenarian siblings and their partners

	Offspring (N=37)	Partners (N=37)	P-value
Levels of miR-663			
ΔCt non-stressed			
Model 1, estimated mean (SE)	-9.69 (0.24)	-9.65 (0.24)	0.82
Model 2, estimated mean (SE)	-9.73 (0.25)	-9.70 (0.25)	0.87
ΔCt stressed			
Model 1, estimated mean (SE)	-9.59 (0.23)	-9.53 (0.23)	0.70
Model 2, estimated mean (SE)	-9.61 (0.23)	-9.57 (0.24)	0.81
Ratio of induction upon stress			
Log2 fold change stressed to non-stressed			
Model 1, estimated mean (SE)	0.24 (0.13)	0.31 (0.13)	0.70
Model 2, estimated mean (SE)	0.24 (0.14)	0.32 (0.14)	0.67

SE: standard error, ΔCt: delta cycle threshold. Calculations were made with the ΔΔCt method, in which Ct values of microRNA-663 were normalised to those of a housekeeper gene. Higher ΔCt values indicate higher microRNA-663 expression. The log2 fold change was calculated as miR-663 expression levels in stressed to those in non-stressed conditions. Number of cardiovascular diseases include cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus. Donors with a datapoint 3 standard deviations below or above the mean were excluded from this analysis.
Model 1: adjusted for batch and repeated measurements
Model 2: adjusted for batch, repeated measurements, gender, chronological age and number of cardiovascular diseases

in donors with and in donors without cardiovascular diseases. This stratification on cardiovascular disease did not materially alter the results, however, significance decreased due to the lower sample size (Table 3).

Next we questioned whether the levels of miR-663 in stressed and non-stressed conditions and the ratio of stress induction differed in middle aged offspring from nonagenarian siblings compared to their partners of the same chronological age. As shown in table 4, the offspring and partners did not differ in their mean miR-663 level either in stressed or non-stressed conditions or in their ratio of miR-663 induction upon stress. Adjustment for possible confounders did not change these results.

Discussion

While no differences depending on chronological age categories in non-stressed conditions were observed, mean miR-663 levels in stressed conditions were dependent on chronological age categories, being higher at higher age. Furthermore, the ratio of miR-663 induction upon stress was significantly associated with chronological age categories. No association of miR-663 and biological age was found when comparing middle aged offspring of nonagenarian siblings with their partners.

Single miRNA's can potentially downregulate several mRNA targets, and so far few targets of miR-663 have been validated: renin and ApoE [21], p21 [22], JunB and JunD [23] and TGFβ1 [24]. The exact mechanism by which miR-663 could act in (cellular) aging therefore remains to be elucidated. Previously higher levels of miR-663 in both replicative and stress-induced senescence *in vitro* were observed [10;11]. Furthermore, higher levels of miR-663 were seen in fibroblasts and memory T-cells that were derived from older donors compared to younger donors [12]. We did not find differences in levels of miR-663 dependent on chronological age categories in non-stressed conditions, which could possibly be explained by different age ranges of donors (especially the inclusion of adolescents in comparison to children). Our group has previously shown that fibroblasts from donors of different age have different stress-induced increases in markers of senescence and apoptosis [4]. In line with these findings we showed that fibroblasts from donors of different age categories respond differently in their miR-663 level upon cellular stress *in vitro*.

Stress-induced differences in senescence and apoptosis were also observed in middle aged offspring of nonagenarian siblings and their partners of different biological age [4]. To our knowledge we are the first reporting on levels of miR-663 and miR-663 stress induction

dependent on the propensity for familial longevity, and show that both levels of miR-663 and its stress induction are not dependent on this propensity. Another factor that reflects biological age is the presence of age-related pathologies. A possible relation between cardiovascular diseases and miR-663 has been reported in few studies. One study showed that miR-663 levels are lower in kidney tissue form hypertensive donors than in tissue of normotensive donors [21]. MiR-663 has furthermore been (indirectly) related to the process of atherosclerosis [25;26]. Stratification on donors with and without cardiovascular diseases however did not alter our results.

Recently miR-663 was shown to be involved in the induction of ATF4 and the downregulation of VEGF in HUVECs by several unfolded protein response inducers and oxidized lipids, providing a possible mechanism in atherosclerosis [26]. Also another factor in development of atherosclerosis, oscillatory shear stress, was associated with an upregulation of miR-663 in HUVECs [25]. In another study miR-663 levels in tumor tissue were found to be increased with longer ischemia time and evidence was found that miR-663 affected stress response through FOSB [27]. Following TDP-43 depletion an upregulated miR-663 expression and a decrease of epoxide hydrolase, an antagonist of oxidative stress and a possible target of miR-663, were observed too [28]. Levels of miR-663 were observed to be higher in denatured dermis in deep burn wounds compared to normal dermis [29]. In a study on the effect of 4-hydroxynonenal, a lipid peroxidation product affecting cell growth and differentiation, on microRNA expression a significant upregulation of miR-663 was observed [30]. All these examples would support the idea that miR-663 is induced upon various stressors. Considering the involvement of miR-663 in replicative and stress-induced senescence and its relation with various physiological stressors, we hypothesize that miR-663 influences cell cycle arrest after encountering cellular stress, however this hypothesis remains speculative in nature. Indeed, miR-663 was observed to act as a tumor suppressor by decreasing the proliferation of human gastric cancer cells both *in vitro* as *in vivo* [31]. Also, the antioxidant resveratrol with possible antitumorigenic properties was found to impair the oncogenic miR-155 through upregulation of miR-663 [23]. However, in contrast to our hypothesis oncogenic properties of miR-663 in nasopharyngeal cancer cell lines have been described as well [22]. In this study, miR-663 was shown to target p21 and herewith induce cell cycle progression.

One of the strengths of this study is the large number of human donors that were included. These donors are part of an extensively phenotyped cohort, fibroblasts from their skin biopsies were grown under highly standardized conditions. A limitation of the study is the cross-sectional design, which does not allow for the observation of a causal relation. Furthermore we only measured miR-663 and none of its potential targets. Therefore, we cannot elucidate whether higher stress-induced levels of miR-663 are a mere stress response, a by-product of stress-

induced senescence, or plays a part in one of the senescence pathways. Also, while treatment of fibroblasts with rotenone increases ROS levels, SAβgal and decreases growth rate [32], it was shown that the stress-induced senescence by rotenone is not conditionally dependent on the generation of ROS [33]. Therefore, the observed differences could be particular to rotenone as a stressor, and not necessarily dependent on increased ROS (in contrast to other stressors such as hydrogen peroxide). We tested our hypothesis in a model of human dermal fibroblasts, it could be that miR-663 induction upon stress and its relation with aging is specific for human dermal fibroblasts and is not universally present in other cell types. While we observe that chronological age of donors and in vitro miR-663 induction upon stress are associated, it of course remains an interesting but unresolved questions whether this phenomenon has in vivo consequences.

In conclusion, we have shown the association of miR-663 levels upon stress with chronological age categories in human dermal fibroblasts. Future investigations should focus on the targets of miR-663 and the causality of this association to strengthen these findings.

Acknowledgements

We would like to thank Corine de Koning-Treurniet, Joke Blom and Pim Dekker for their work in the laboratory.
This work was supported by the Austrian Science Fund [P 24498-B20]; Genome Research Austria GEN-AU [Project 820982 „Non-coding RNAs"], grants by the Herzfelder'sche Familienstiftung to RGV and CE.R.I.E.S to JG, SenterNovem, IGE01014 and IGE5007, NGI/NWO; 05040202 and 050-060-810.

References

(1) Schoenmaker M, de Craen AJ, de Meijer PH et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. *Eur J Hum Genet* 2006;14:79-84.

(2) Westendorp RG, van HD, Rozing MP et al. Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: The Leiden Longevity Study. *J Am Geriatr Soc* 2009;57:1634-1637.

(3) Wijsman CA, Rozing MP, Streefland TC et al. Familial longevity is marked by enhanced insulin sensitivity. *Aging Cell* 2011;10:114-121.

(4) Dekker P, Maier AB, van HD et al. Stress-induced responses of human skin fibroblasts in vitro reflect human longevity. *Aging Cell* 2009;8:595-603.

(5) Waaijer ME, Parish WE, Strongitharm BH et al. The number of p16INK4a positive cells in human skin reflects biological age. *Aging Cell* 2012;11:722-725.

(6) Baker DJ, Wijshake T, Tchkonia T et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 2011;479:232-236.

(7) Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet* 2009;10:94-108.

(8) Chen LH, Chiou GY, Chen YW, Li HY, Chiou SH. MicroRNA and aging: a novel modulator in regulating the aging network. *Ageing Res Rev* 2010;9 Suppl 1:S59-S66.

(9) Grillari J, Grillari-Voglauer R. Novel modulators of senescence, aging, and longevity: Small non-coding RNAs enter the stage. *Exp Gerontol* 2010;45:302-311.

(10) Marasa BS, Srikantan S, Martindale JL et al. MicroRNA profiling in human diploid fibroblasts uncovers miR-519 role in replicative senescence. *Aging (Albany NY)* 2010;2:333-343.

(11) Maes OC, Sarojini H, Wang E. Stepwise up-regulation of microRNA expression levels from replicating to reversible and irreversible growth arrest states in WI-38 human fibroblasts. *J Cell Physiol* 2009;221:109-119.

(12) Hackl M, Brunner S, Fortschegger K et al. miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging. *Aging Cell* 2010;9:291-296.

(13) Bootsma-van der Wiel A, Gussekloo J, de Craen AJ, Van EE, Bloem BR, Westendorp RG. Common chronic diseases and general impairments as determinants of walking disability in the oldest-old population. *J Am Geriatr Soc* 2002;50:1405-1410.

(14) Maier AB, le CS, de Koning-Treurniet C, Blom J, Westendorp RG, van HD. Persistence of high-replicative capacity in cultured fibroblasts from nonagenarians. *Aging Cell* 2007;6:27-33.

(15) Dekker P, de Lange MJ, Dirks RW et al. Relation between maximum replicative capacity and oxidative stress-induced responses in human skin fibroblasts in vitro. *J Gerontol A Biol Sci Med Sci* 2011;66:45-50.

(16) Li N, Ragheb K, Lawler G et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 2003;278:8516-8525.

(17) Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.

(18) Lardizabal MN, Nocito AL, Daniele SM, Ornella LA, Palatnik JF, Veggi LM. Reference genes for real-time PCR quantification of microRNAs and messenger RNAs in rat models of hepatotoxicity. *PLoS One* 2012;7:e36323.

(19) Sharbati-Tehrani S, Kutz-Lohroff B, Bergbauer R, Scholven J, Einspanier R. miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. *BMC Mol Biol* 2008;9:34.

(20) Son DJ, Kumar S, Takabe W et al. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. *Nat Commun* 2013;4:3000.

(21) Marques FZ, Campain AE, Tomaszewski M et al. Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. *Hypertension* 2011;58:1093-1098.

(22) Yi C, Wang Q, Wang L et al. MiR-663, a microRNA targeting p21(WAF1/CIP1), promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma. *Oncogene* 2012.

(23) Tili E, Michaille JJ, Adair B et al. Resveratrol decreases the levels of miR-155 by upregulating miR-663, a microRNA targeting JunB and JunD. *Carcinogenesis* 2010;31:1561-1566.

(24) Liu ZY, Zhang GL, Wang MM, Xiong YN, Cui HQ. MicroRNA-663 targets TGFB1 and regulates lung cancer proliferation. *Asian Pac J Cancer Prev* 2011;12:2819-2823.

(25) Ni CW, Qiu H, Jo H. MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. *Am J Physiol Heart Circ Physiol* 2011;300:H1762-H1769.

(26) Afonyushkin T, Oskolkova OV, Bochkov VN. Permissive role of miR-663 in induction of VEGF and activation of the ATF4 branch of unfolded protein response in endothelial cells by oxidized phospholipids. *Atherosclerosis* 2012.

(27) Borgan E, Navon R, Vollan HK et al. Ischemia caused by time to freezing induces systematic microRNA and mRNA responses in cancer tissue. *Mol Oncol* 2011;5:564-576.

(28) Buratti E, De CL, Stuani C, Romano M, Baralle M, Baralle F. Nuclear factor TDP-43 can affect selected microRNA levels. *FEBS J* 2010;277:2268-2281.

(29) Liang P, Lv C, Jiang B et al. MicroRNA profiling in denatured dermis of deep burn patients. *Burns* 2012;38:534-540.

(30) Pizzimenti S, Ferracin M, Sabbioni S et al. MicroRNA expression changes during human leukemic HL-60 cell differentiation induced by 4-hydroxynonenal, a product of lipid peroxidation. *Free Radic Biol Med* 2009;46:282-288.

(31) Pan J, Hu H, Zhou Z et al. Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells. *Oncol Rep* 2010;24:105-112.

(32) Dekker P, van Baalen LM, Dirks RW et al. Chronic inhibition of the respiratory chain in human fibroblast cultures: differential responses related to subject chronological and biological age. *J Gerontol A Biol Sci Med Sci* 2012;67:456-464.

(33) Goncalves AP, Maximo V, Lima J, Singh KK, Soares P, Videira A. Involvement of p53 in cell death following cell cycle arrest and mitotic catastrophe induced by rotenone. *Biochim Biophys Acta* 2011;1813:492-499.