MicroRNA 299-3p modulates replicative senescence in endothelial cells

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Contribution
Conceived and designed the experiments: MRM SAB PFW. Performed the experiments: HLJ. Analyzed the data: HLJ PMV PFW. Contributed reagents/materials/analysis tools: MRM SAB PFW. Drafted the paper: HLJ PFW. Critical reviewed: PMV MRM SAB.

Suggested running title
MicroRNA 299-3p and endothelial senescence
Abstract

MicroRNAs (miRNAs) regulate various cellular processes. While several genes associated with replicative senescence have been described in endothelial cells, miRNAs that regulate these genes remain largely unknown. The present study was designed to identify miRNAs associated with replicative senescence and their target genes in human umbilical vein endothelial cells (HUVECs). An integrated miRNA and gene profiling approach revealed that hsa-miR-299-3p is up-regulated in senescent HUVECs compared to the young cells and one of its target genes could be IGF1. IGF1 was up-regulated in senescent compared to young HUVECs and knockdown of hsa-miR-299-3p dose-dependently increased the mRNA expression of IGF1, more significantly observed in the pre-senescent cells (passage 19) compared to the senescent cells (passage 25). Knockdown of hsa-miR-299-3p also resulted in significant reduction in the percentage of cells positively stained for senescence associated-beta-galactosidase, increased in cell viability measured using MTT assay but marginal increases in cell proliferation and cell migration capacity measured by real-time growth kinetics analysis. Moreover, knockdown of hsa-miR-299-3p also increased proliferation of cells treated with H2O2 to induce senescence. These findings suggest that hsa-miR-299-3p may delay or protect against replicative senescence by improving the metabolic activity of the senesced cells but does not stimulate growth of the remaining cells in senescent cultures. Hence, these findings provide an early insight into the role of hsa-miR-299-3p in the modulation of replicative senescence in HUVECs.

Keywords

MicroRNA; Aging; hsa-miR-299-3p; IGF1
Introduction

Normal cells have a limited lifespan in which they cease to proliferate after a finite number of population doublings (55). Replicative senescence is achieved when the cells enter the irreversible growth arrest phase ($G_0/G_1$ arrest) of the cell cycle (40). Senescent cells do not divide but remain viable and metabolically active. These cells have enlarged and flattened morphology, increased granularity and vacuolization, altered expressions of genes and proteins and shortened telomere length; they characteristically express senescence associated-$\beta$-galactosidase (SA-$\beta$-gal) activity at pH 6.0 (22). Besides replicative senescence, numerous factors can contribute to stress-induced premature senescence. They include exposure to sub-lethal dose of DNA damaging agents (e.g. certain anticancer drugs), gamma irradiation, oxidative stress, over-expression of activated oncogenic Ras (or of its downstream effectors such as Raf), activation of tumor suppressor genes (e.g. $p53$, $p16$ and $pRB$), deprivation of nutrients or growth factors and improper cell contacts (4, 12).

MicroRNAs (miRNAs) are a well-recognized group of short ($\approx 22$ nt), non-coding RNAs (27). Currently, the miRNA database [Sanger miRBase (http://www.mirbase.org/)] lists more than eighteen thousand entries. miRNAs regulate gene expression by targeting 3’ untranslated region (UTR) of mRNA transcripts which in turn results in translational inhibition or degradation of mRNA. The majority of miRNAs repress gene expression but a minority group activates it by up-regulating translation (39, 54). Unique alterations in miRNA expression contribute to the regulation and induction of diseases such as various types of cancers, diabetes and autoimmune diseases (31). In particular, the miRNA profile is altered in heart disease and the pattern of miRNA expression is distinct in different forms of cardiac disorders (19). A unique miRNAs signature is associated with the involvement of specific signal transduction pathway and thus
with the prognosis and pathogenesis of the disease. For instance, miR-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts and miR-126 accelerates the development of atherosclerosis (3, 51). In addition, over-expression of miR-34a induces cell cycle arrest and senescence in human umbilical vein endothelial cells (HUVEC), an effect mediated by the SIRT1-p53 pathway (59).

Endothelial cells are specialized cells that line the vascular lumen. They play an important role in modulating vascular tone, exchanges of molecules between blood and tissues, prevention of coagulation and formation of new blood vessels. Senescent endothelial cells contribute to age-related vascular disorders such as atherosclerosis and cardiovascular diseases (36). Alterations in miRNA expression have been reported in senescent endothelial cells, suggesting that miRNAs play roles in the regulation of senescence in these cells (35, 53). HUVECs have been widely used in numerous in vitro studies on human endothelial cells. Despite discrepancies noted between HUVECs and the different parts of vasculature, they do share many common functional and morphological features (50, 53). Hence, results obtained from HUVECs will provide preliminary observations leading to subsequent physiologically relevant in depth investigations.

In earlier studies in senescent HUVECs, miRNA and gene expression profiling have been performed independently, making direct identification of miRNA target genes challenging (7, 23, 35, 40, 47). The present study, hence, was designed to perform an integrated study of miRNA and gene expression in HUVECs, in the hope to provide new insights into the role of miRNAs in cellular senescence and age-related disorders and to define their potential as diagnostic markers and/or therapeutic targets.
Materials and methods

Cell culture

HUVECs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained in endothelial cell medium (ECM) supplemented with 5% FBS, 1% endothelial cell growth supplement and 1% of penicillin/streptomycin (ScienCell Research Laboratories), at 37°C in a humidified atmosphere of 95% air/5% CO2. The number of population doublings (PD) was calculated as described (8, 25) using the formula:

\[ PD = \frac{\ln \text{[number of cells harvested]} - \ln \text{[number of cells seeded]}}{\ln 2} \]

Cells were seeded at a constant initial density of 5x10^5 cells per T75 flask and passaged using 0.25% trypsin EDTA every two to three days in order to maintain the cultures in constant log phase. Replicative senescent cells were obtained by consecutively passaging the young cells until the cells proliferation became nearly arrested, approximately at passage 25 (PD ~ 45).

Young and senescent cells used in experiments are cells with PD < 10 and PD > 45 respectively, unless otherwise mentioned. Morphological changes were monitored and evaluated for every subculture. The images of young and senescent cells were captured, examined and recorded with light microscopy at a magnification of 200X.

Senescence-associated beta galactosidase

Cells were stained using a SA-β-gal staining kit (Cell-Signaling Technology, Beverly, MA, USA) following the manufacturer’s instructions. Briefly, cells were washed once in one time (1X) PBS, fixed for 15 minutes in 1X fixative solution, washed twice in 1X PBS before incubated in fresh SA-β-gal staining solution at 37°C overnight. The percentage of positively stained cell (blue cells)
versus total cells was counted by randomly choosing ten microscopic fields under 20X objective magnification.

**MTT Cell Viability Assay**

Briefly, cells were seeded in 96-wells plates at optimum cell density. After 18–24 hours, cells were transfected with 31.25, 62.5, 125, 250 and 500 nM of miRIDIAN hairpin inhibitor of hsa-miR-299-3p (A299-3p) (Dharmacon) for 24 hours. Control wells were transfected with equal concentrations of control antisense oligonucleotide (C_A) i.e. miRIDIAN hairpin inhibitor negative control # 2 (Dharmacon). Following transfection, cells were incubated in the dark with 2 mg/ml MTT at 37°C for 2 hours, then the medium was carefully removed and 100 μl of DMSO was added to dissolve the formazan crystals formed. Absorbance was measured at 570nm in a plate reader. Cell viability was calculated using the following formula: (mean absorbance in test wells)/ (mean absorbance in control well) x 100%.

**H₂O₂ study**

Hydrogen peroxide, H₂O₂ (BDH AnalR, UK) was used to induce premature senescence. Briefly, young cells were seeded at optimum cell density and incubated overnight. The cells were subjected to two successive sub-lethal treatments of 200 μM hydrogen peroxide, H₂O₂ in ECM containing 5% FBS for two hours, with one treatment per day for two consecutive days. At the end of each treatment, the treatment media were removed and the cells were washed twice with 1X PBS and maintained in ECM with 5% FBS.

**Real time growth profile**

Cells were seeded in 16-well E-plate and the growth kinetic of the cells was monitored continuously using Roche xCELLigence Real Time Cell Analyzer (RTCA) (Roche Diagnostic,
Mannheim, Germany) DP instrument at 37 °C in a humidified atmosphere of 95% air/5% CO₂. The xCELLigence system utilizes E-plate (Roche Diagnostic) which contains inter-digitated micro-electrodes on the bottom of the plate that detect local ionic changes as cells proliferate and the proliferation rate is measured as electrode impedance. Cell sensor impedance was expressed as an arbitrary unit termed Cell Index (CI). The CI at each time point was defined as \( \frac{R_n - R_b}{15} \), where \( R_n \) is the cell electrode impedance of the well and the \( R_b \) is the background impedance of the well with the media alone.

**Telomere length measurement**

Relative telomere length was measured using a modified quantitative real time polymerase chain reaction (qRT-PCR) method (6). Genomic DNAs were extracted from cells using QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s protocol. Telomeric hexamer repeats were quantitated using telomere (T) and single copy gene (S) PCR. T and S PCR were performed using Power SYBR green PCR master mix (Applied Biosystems, Foster, CA, USA) on ABI StepOne system. Telomere and beta globin primer pairs (primer sequences shown below) were used for T and S PCR respectively:

- telg: ACACTAAGGTGGGTTGGGTTGGGTTGGGTTAGTGT
- telc: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
- hbgu: CGGCGGCGGGCGGCGGCGGCGGCGGCTGGGGCGGGCTTCATCCACGTTACCTTG
- hbgd: GCCCGGCCCCGCCCCCGGCCCCGTCCCCGCCGAGGAGGAGGAGGACTGCTGCGTT

The thermal cycling profile for both T and S PCR was as follow: Holding stage: 15 minutes at 95°C; Cycling stage I: 2 cycle of 15 seconds at 94 °C, 15 seconds at 49 °C; Cycling stage II: 32
cycles of 15 seconds at 94°C, ten seconds at 62°C, 15 seconds at 74°C; Melt curve stage: 15 seconds at 94°C, one minute at 60°C, 15 seconds at 95°C with gradual increment of 0.3°C.

Standard curves (a plot of \( C_t \) versus log [amount of input DNA]) were established for both T and S PCR using randomly chosen genomic DNA of young cells. Relative telomere lengths of samples were calculated based on the T/S ratio obtained from standard curves.

**miRNA and gene expression microarray**

Once the RS cells were established, analyses of the expressions of 866 human miRNAs and 89 human viral miRNAs, based on Sanger miRNA database release 12.0, and 19,596 human genes were performed using Agilent Human MicroRNAs and Whole Human Genome microarray platforms, respectively. Total RNAs were isolated from cells using miRNeasy mini kit (Qiagen) according to manufacturer’s protocol. RNA integrity of the total RNA was accessed using Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). For microRNA microarrays, total RNAs (100 ng) were labeled with Cyanine 3-pCp and hybridized onto Human MicroRNA Array version 3.0 (8X15K) with 40-60 mer oligonucleotides probe spotted on the slide by SurePrint Inkjet technology (Agilent Technologies Inc.). For gene expression microarrays, cRNA was synthesized from 50 ng of total RNAs, labeled with Cyanine 3-CTP, and hybridized onto Whole Human Genome Array (4x44k) which was spotted by the 60-mer SurePrint technology (Agilent Technologies Inc.). The microarray slides were scanned on microarray Scanner B and data was extracted using Feature Extraction software (Agilent Technologies Inc.). Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession No.GSE37093.
**Microarray data analysis**

Integrated analysis of microRNA and gene expression microarray data was performed using GeneSpring GX version 11.05 (Agilent Technologies Inc). Threshold of raw signals were set at 1.0. The normalization algorithm employed was quantile and baseline transformation to median of all samples was performed. Differential miRNA and gene expression profiles of young versus senescent cells were generated using Volcano Plot in GeneSpring GX which consists of the significance analysis and fold change filter. Significance analysis was performed using Student’s t-test for unpaired observations with asymptotic corrected P-value < 0.05 and fold change filter of > 2.0. The multiple testing correction employed was Benjamini-Hochberg. The TargetScan program integrated in GeneSpring was used to identify the biological targets of the differentially expressed miRNAs. In addition, homology regions between miRNA of interest and 3’UTR of target genes of interest were identified using miRanda (http://www.microrna.org/microrna/home.do). The miRNA profile was then translated to the gene expression data. The joint miRNA and gene expression profile was then subjected to pathway analysis. The pathway map generated from pathway analysis was analyzed to select miRNAs of interest. The miRNAs of interest reported have not been previously associated with cellular senescence but showed association with known cellular senescence-associated genes in the pathway maps.

**qRT-PCR validation of miRNA and gene expression**

Two-step qRT-PCR was performed to quantitatively measure the expression of miRNA and gene in order to validate the microarray data. For validation of miRNA expression, cDNA was synthesized from ten nanograms of total RNA using a Taqman microRNA Reverse Transcription
Kit according to manufacturer’s protocol (Applied Biosystem). qRT-PCR was performed using Taqman MicroRNA Assay and Taqman Universal PCR Master Mix (Applied Biosystem). Taqman MicroRNA Assay ID are as follow: hsa-miR-130b (assay ID: 000456), hsa-miR-18b (assay ID: 002217), hsa-miR-299-3p (assay ID: 001015), hsa-miR-338-3p (assay ID: 002252), hsa-miR-134 (assay ID: 001186), hsa-miR-17 (assay ID: 002308), hsa-mir-188-5p (assay ID: 002320), RNU6B (control miRNA assay) (assay ID: 001093). RNU6B (human) was used as endogenous reference RNA for normalization of data. For validation of gene expression, cDNA was synthesized from 250 ng total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the manufacturer’s protocol. qRT-PCR was performed using Taqman Gene Expression Assay and Taqman Fast Advanced PCR Master Mix. Taqman Gene Expression Assay ID are as follow: IGF1 (Assay ID: Hs_01547656_m1), SLFN13 (Assay ID: Hs_00431187_m1), HDAC9 (Assay ID: Hs_00206843_m1). GAPDH (Assay ID: 4333764F) was used as endogenous reference RNA for normalization of data. Analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

miRNA knockdown study

Transfection of HUVECs was performed using DharmaFECT 1 according to the manufacturer’s instructions (Dharmacon, Lafayette, CO, USA). Briefly, cells were seeded in growth medium without antibiotics at a density of 10,000 cells per well and incubated overnight. The cells were then transfected with 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 nM of miRIDIAN hairpin inhibitor of hsa-miR-299-3p (A299-3p) (Dharmacon) for 24 hours. After the knockdown of miRNA, Taqman MicroRNA Assay was performed as described above to measure the expression of hsa-miR-299-3p. The expression of hsa-miR-299-3p was compared to growth medium control.
For mRNA expression measurement, the cells were transfected with 31.25, 62.5, 125, 250 and 500 nM of miRIDIAN hairpin inhibitor of hsa-miR-299-3p (A299-3p) (Dharmacon) for 24 hours. Control wells were transfected with equal concentrations of control antisense oligonucleotide (Cₐ) i.e. miRIDIAN hairpin inhibitor negative control # 2 (Dharmacon). After the knockdown of miRNA, Taqman Gene Expression Assay was performed as described above to measure the expression of IGF1, HDAC9 and SLFN13 mRNA.

For SA-β-gal staining, real time growth profile and cell migration assay, the cells were transfected with 31.25, 62.5, 125, 250 and 500 nM of miRIDIAN hairpin inhibitor of hsa-miR-299-3p (A299-3p) (Dharmacon) for 48 hours. Control wells were transfected with equal concentrations of control antisense oligonucleotide (Cₐ) i.e. miRIDIAN hairpin inhibitor negative control # 2 (Dharmacon). SA-β-gal staining, real time growth study, cell cycle analysis, total nitric oxide measurement, cell migration assay were performed to determine the percentage of SA-β-gal positively stained cells, growth, migration capacity and total NO production after transfection.

For concurrent knockdown of multiple miRNAs, cells were transfected with a total concentration of 31.25, 62.5, 125, 250 and 500 nM of combination of miRIDIAN miRNA hairpin inhibitor for hsa-miR-134, -299-3p and -338-3p (simplified as “comb” hereafter) for 24 hours. For instance, 500 nM of comb means a pool of 167 nM of hsa-mir-134, -299-3p and -338-3p inhibitor, respectively. Control wells were transfected with equal concentrations of control antisense oligonucleotide (Cₐ) i.e. miRIDIAN hairpin inhibitor negative control # 2 (Dharmacon). Total RNA were harvested after transfection and Taqman Gene Expression Assay was performed as described above to check the expression of IGF1 mRNA.

**Cell migration assay**
Cell migration assay was performed using CIM plates (Roche Diagnostic) according to the standard protocol suggested by the manufacturer. Briefly, after 48 hours transfection, cells were starved using 0.5% FBS containing growth media for five hours. Following starvation, they were trypsinized and seeded onto upper chamber of CIM plates at cell density of 14,000 cells per well. Lower chamber of CIM plates were filled up with 10% FBS containing growth media acting as chemoattractant. CIM plates were loaded onto RTCA DP instrument (Roche Diagnostic) to monitor real time cell migration. Microelectronic sensors are integrated onto the underside of the microporous polyethylene terephthalate (PET) membrane of the CIM plate (Boyden-like chamber). Cells migrate from the upper chamber through the membrane into the bottom chamber, contact and adhere to the electronic sensors on the underside of the membrane and alter impedance. Cell sensor impedance was expressed as Cell Index (CI).

**Statistical analysis**

All data are presented as means ±SEM. Statistical analysis was performed with Student’s t-test using GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA). P-values less than 0.05 were accepted to indicate statistically significant differences. Statistical significance is expressed as ***, P < 0.001; **, P < 0.01; *, P < 0.05.

**Results**

**Characterization of replicative senescence**

Replicative senescence (RS) was confirmed in HUVECs with more than 45 population doublings by the following observations: a] RS cells were relatively enlarged, flattened and showed an increase in granulation and vacuolization as compared to their younger counterpart (Figure 1A); b] RS showed a significant increase (~ 60%) in the percentage of SA-β-gal positive
cells compared to young cells (Figure 1B and 1C); c] The real time growth kinetics of RS showed a relatively lower cell index and remained viable in culture over a long period of time while young cells showed a relatively higher cell index and were eventually eliminated from the culture (Figure 1D); d] Cell cycle analysis revealed that RS cells had a significant increase in G0/G1 population (~40%) when compared to the young cells, suggesting that RS cells stopped proliferation through G0/G1 arrest in the cell cycle (Figure 1E); and e] The telomere length of RS cells was also shorter compared to the young cells (Figure 1F).

**miRNA and gene expression profile**

The comparison of the miRNA profile in young and RS cells showed that 29 miRNAs were expressed differentially in the latter, including up-regulation of 19 miRNAs and down-regulation of ten others (Table 1). In the gene expression analysis, 251 genes were differentially regulated in RS, with 130 down- and 121 up-regulations (Figure 2A). Microarray datasets are available for viewing at GEO through accession No.GSE37093. To validate the microarray findings, qRT-PCR was performed for seven miRNAs. Overall, the qRT-PCR analysis showed satisfactory correlation with the microarray results, albeit smaller fold-changes were found by qRT-PCR (Figure 3A). In particular, the qRT-PCR results showed that hsa-miR-130b, -18b, and -17 were down-regulated while hsa-miR-299-3p, -338-3p, -134, and -188-5p were up-regulated in RS compared to young cells (Figure 3A). Computational simulation of biological interactions of differentially expressed target genes of hsa-miR-299-3p in senescent HUVECs were also identified (Figure 2C). In additional, TargetScan analysis suggested that IGF1 is a target of hsa-miR-299-3p, -134 and 338-3p. Homology region between hsa-miR299-3p and 3’UTR of IGF1 was also identified by miRanda, where the score of homology match (mirSVR score) was -
0.4165 [Figure 2B(i)]. miRNA targets with mirSVR score < −0.1 is considered as “good” and miRNA targets with > −0.1 mirSVR score indicates “non-good” (46). This further confirmed the potential regulation of IGF1 by hsa-miR-299-3p. Prior to subsequent knockdown studies, the inherent mRNA expressions of IGF1. The qRT-PCR results showed that IGF1 was up-regulated by approximately seven-fold in RS compared to young cells (Figure 3B).

**IGF1 as potential target of hsa-miR-299-3p**

hsa-miR-299-3p was knocked down transiently in RS cells by transfection with a range of concentrations (7.8 to 500 nM) of miRNA hairpin inhibitor for hsa-miR-299-3p (A299-3p) or control antisense oligonucleotide (CA). Target genes expression, percentage of SA-ß-gal positive, cell growth and migration were determined after the knockdown. Transfection with the hsa-miR-299-3p inhibitor, A299-3p resulted in a significant concentration-dependent knockdown, ranging from 45% (7.8 nM) to 85% (500nM) decrease in the expression of hsa-miR-299-3p as quantified by qRT-PCR (Figure 3C).

To investigate if hsa-miR-299-3p targets IGF1, the mRNA expression level of IGF1 was measured following the knockdown of hsa-miR-299-3p. Transient inhibition of hsa-miR-299-3p resulted in a dose-dependent increase in the mRNA expression of IGF1, with the level significantly surpassed that of the antisense oligonucleotide at 250nM (Figure 4A; p<0.01) by approximately 30% when compared to the control antisense oligonucleotide. Since the knockdown was performed on near arrested cell at passage 25 (PD ~ 45), the true effect of hsa-miR-299-3p inhibition may not be apparent. Hence, we used cells which are at the pre-senescence stage at passage 19 cells for subsequent hsa-miR-299-3p knockdown. In passage 19
cells, knockdown of hsa-miR-299-3p resulted in a dose-dependent (except at 500 nM) increase in the mRNA expression of IGF1 (Figure 4B). Significant increase was observed at much concentrations of hsa-miR-299-3p inhibitor, A299-3p (62.5 nM onwards). Hence, this result showed that knockdown of hsa-miR-299-3p affects IGF1 mRNA expression.

Because TargetScan analysis identified that hsa-miR-134 and -338-3p also target IGF1, we hypothesize that knockdown of hsa-miR-299-3p in combination with hsa-miR-134 and -338-3p may enhance the elevation in IGF1 mRNA expression. Knockdown of hsa-miR-299-3p in combination with hsa-miR-134, and -338-3p resulted in significant increase in the mRNA expression of IGF1 when compared to the control antisense oligonucleotide. Concurrent inhibition of hsa-miR-134, 299-3p and -338-3p at 31.25 nM and 62.5 nM result in ~130% and ~170% increase in the mRNA expression of IGF1, respectively (Figure 4C; p < 0.01 and p < 0.05, Student’s T-test). These data showed that knockdown of hsa-miR-299-3p together with other miRNAs that target IGF1 can release the suppression of IGF1 with greater efficacy at low concentrations of inhibitors used.

**Inhibition of hsa-miR-299-3p and replicative senescence**

To further confirm the role of hsa-miR-299-3p in modulating senescence, SA-β-gal pH 6.0 staining, MTT cell viability assay, real time growth studies and cell migration assay were performed after inhibition of hsa-miR-299-3p at a range of concentration of the hsa-miR-299-3p inhibitor, A299-3p. A significant dose-dependent decrease in the percentage of SA-β-gal positive cells was observed at all concentrations of inhibition of hsa-miR-299-3p when compared to $C_A$ except for 500 nM (Figure 5A and 5B). On the average, RS cultures transfected with $C_A$
contained approximately 40% SA-β-gal positive cells. The greatest reduction in percentage of SA-β-gal positive cells (reduced from ~43% to 22%) was observed at 31.25 nM of inhibition of hsa-miR-299-3p (Figure 5B), while the lowest reduction in percentage of SA-β-gal positive cells (reduced from ~40% to 30%) was observed at 500 nM of inhibition of hsa-miR-299-3p which did not reach statistical significance (Figure 5B). Cell viability as measured by MTT assay also showed a significant dose dependent increase in cell viability following the inhibition of hsa-miR-299-3p at all tested concentrations when compared to C_A except for 500 nM (Figure 5C). Real-time growth kinetics of RS cells was examined following knockdown of hsa-miR-299-3p (Figure 6A-E). Normalized cell index of RS cells increased after knockdown of hsa-miR-299-3p, particularly at 250nM (Figure 6D) and 500 nM (Figure 6E), though did not reach statistical significance. This suggests that knockdown of hsa-miR-299-3p marginally improved the proliferative capacity of RS cells. Since knockdown of hsa-miR-299-3p could only marginally improve the proliferative capacity of RS cells, we investigated whether knockdown of hsa-miR-299-3p could protect cells from H_2O_2-induced senescence by treating the cells with H_2O_2 followed by transfection with hsa-miR-299-3p inhibitor, A299-3p. Real-time growth kinetics analysis showed that transfection with 62.5 and 125 nM of hsa-miR-299-3p inhibitor, A299-3p resulted in significant increase in the proliferation of the transfected cells compared to those transfected with negative control inhibitor, C_A (Figure 7B and 7C; p < 0.01 and p < 0.05, Student’s T-test). This finding suggests that inhibition of hsa-miR-299-3p can overcome H_2O_2-induced senescence and stimulate the proliferation of the senescent cells.

To evaluate the role of hsa-miR-299-3p in modulating cell migration of RS cells, cell migration assay was performed following knockdown of hsa-miR-299-3p (Figure 8A-E). Cell index of RS
cells increased at 62.5 nM (Figure 8B) and 125 nM (Figure 8C) of A299-3p, though not statistically significant. This indicates that knockdown of hsa-miR-299-3p at 62.5 nM and 125 nM slightly increase the cell migration capacity of RS cells.

Discussion

The involvement of several miRNAs is established in the regulation of replicative senescence. These include hsa-miR-146a [targeting NOX4 (53)], hsa-miR-34a [acting on the SIRT1-p53 pathway (59)] and hsa-miR-17 [targeting the cdk inhibitor p21/CDKN1A (15)]. Because of the rapid expansion of the field of miRNAs and the complexity of the interaction of individual miRNAs with targeting several genes, there is a need to delineate the role of specific miRNAs in replicative senescence. Hence, the present study undertook an integrated profiling analysis of miRNAs and genes in senescent HUVECs to identify other potential senescence-associated miRNAs. Changes in gene expression profiles associated with senescence have been reported in several cell types (12, 32, 41, 47). In the present study, integrative analysis of miRNA and gene expression profile of RS in HUVECs suggests that 29 miRNAs in RS may regulate at least 251 genes. In particular, the miRNA profiling results reveal that hsa-miR-299-3p is up-regulated in RS HUVECs and TargetScan analysis on gene expression profile derived from the same RNA samples show that one of its potential targets is IGF1. Other highly deregulated miRNAs shown in the miRNA profile was not chosen for further investigation because these have been reported for their association with senescence. For instance, Watanabe et al. showed that hsa-miR-150 induce senescence in NK/T-cell lymphoma lines (57). hsa-miR-146a was reported to modulate endothelial cell aging via NOX4. (53). hsa-miR-299-3p was chosen for
further investigation due to its relevant target gene i.e. IGF1 which was reported to be highly associated with senescence or cell proliferation and growth.

To evaluate the effect of inhibition of hsa-miR-299-3p on replicative senescence, SA-β-gal assay was used. SA-β-gal activity has been widely used as biomarker of senescence in vitro and in vivo since its first description by Dimri et al. (10). It has been described as the “gold standard” marker for cellular senescence, despite the fact that its function and origin remained unknown (9, 14, 38, 56). Studies have shown that increase in SA-β-gal activity in cellular senescence reflects an increase in lysosomal mass in senescent cells and SA-β-gal is not required for senescence (24, 26). The MTT assay is commonly used to screen compounds for effects on viability and reduction of MTT to formazan is due to cellular enzymatic activity in mitochondria, endosomes, and lysosomes (5, 30). Factors in the cell culture environment such as pH and glucose, NADH, or NADPH supply also influence MTT reduction to formazan (17, 34) and thus MTT data also reflect changes in cell metabolism. Knockdown of hsa-miR-299-3p reduced the percentage of SA-β-gal positive cells in the senesced culture and increased cell viability as measured with MTT assay but the real-time growth kinetics data did not show significant increase in cell proliferation. As both SA-β-gal and MTT assay are dependent on cellular enzymatic activities which are reflective of cell metabolism, it is suggestive that knockdown of hsa-miR-299-3p may have improved the metabolic activity of the senescent cells, without stimulating cell proliferation and increase cell numbers. Cell cycle analysis supports this observation as inhibition of hsa-miR-299-3p did not stimulate the G0/G1 population to reenter the cell cycle (data not shown). However, the role of hsa-miR-299-3p in HUVECs senescence is ascertained when knockdown of hsa-miR-299-3p could stimulate proliferation of cells treated with H2O2 to induce senescence. The fact that inhibition of hsa-miR-299-3p can overcome
H$_2$O$_2$-induced senescence and stimulate the proliferation of the senescing cells suggests that hsa-miR-299-3p has a role in senescence and its inhibition can delay premature senescence in HUVECs.

mRNA expression of IGF1 that was up-regulated in RS HUVECs but not in their young counterpart was also elevated following the inhibition of hsa-miR-299-3p in senescent and pre-senescent HUVECs. Moreover, in pre-senescent cells, dose-dependent increased in IGF-1 expression was observed at low concentrations of anti-miR used, suggesting that hsa-miR-299-3p may in part, mediates its actions through IGF1. In addition, knockdown in combination with two other miRNAs, i.e. hsa-miR-134 and -338-3p that target IGF-1 further increased the elevation of IGF1 mRNA expression at low concentrations of anti-miR used. IGF1 signaling has been described as an evolutionarily conserved mechanism of longevity in nematodes, fruit flies, yeast, rodents and human (2). However, a recent contrasting review suggests that IGF1 signaling is not part of an evolutionary conserved mechanism of longevity in human. Life span of human is not increased corresponding to decline in IGF1 signaling, contrary to evidences found in nematodes, fruit flies and rodents [reviewed in (49)]. IGF1 plays an essential role in stimulating DNA synthesis, amino acid uptake, protein synthesis and glucose transport in various cells (2). It also acts as a growth factor in many tissues and tumors (1). Excess of IGF1 predispose human to the risk of neoplastic diseases (49). Contrary, IGF1 deficiency increases the risk for cardiovascular complications in aged individuals, particularly atherosclerosis (18, 52). Despite the fact that IGF1 has been largely associated with aging, the molecular mechanism underlying its action remains controversial. Senescent cells do not respond to IGF1 (in combination with other growth factors) with DNA synthesis and mitosis, despite possessing a comparable number of IGF1 receptors to that of young cells (45). Treatment with IGF1 extends longevity in a mouse
model of premature aging by restoring somatotrophic function (33) and localized IGF1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle (37). There is limited information on the role of miRNAs in modulating IGF1 and in turn regulating senescence. Thus far, it is only reported that mir-71 and mir-239 promote longevity of C. elegans via the insulin/IGF-1-like pathway (42). In addition, the findings of the present study indicate the potential of hsa-miR-299-3p in modulating IGF1 and thus regulating senescence.

In terms of cell migration, although in general we do not see a significant improvement in cell migration following hsa-mir-299-3p knockdown, the knockdown improved migration capacity. Previous reports demonstrated that IGF1 stimulated endothelial cell migration (20, 29, 48). Those findings support the suggestion that cell migration improvement is due to the optimal IGF1 expression modulated by hsa-miR-299-3p.

miRNA expression profiles have been demonstrated to be highly tissue, organ, diseases or developmental stages specific. Numerous studies have been performed to investigate the age-related alteration of miRNA signature in various tissues or organs, aim to identify unique miRNA signature which could be useful for the development of tissue or organ specific biomarkers as well as therapeutic strategies for age-related complications (13, 28, 31, 43, 44, 58). The miRNA signature obtained in present study is in agreement with findings from earlier studies. For instances, hsa-miR-134 [senescent fibroblast], (11), hsa-miR-17 [senescent human diploid fibroblast, HUVEC, renal proximal tubular epithelial cells, T-cells, bone marrow derived mesenchymal stem cells, human foreskin] (16), hsa-miR-146a [senescent HUVEC] (53) were found to be differentially expressed in RS HUVECs in present study. The similarities suggest that those miRNAs are potential biomarker of cellular senescence due to its high reproducibility across various independent studies. Furthermore, the similarities strengthen the reliability of
current findings. On the other hand, differences in miRNA expression profile between current
study and previous studies were also noticed. hsa-miR-217 (35) and -34a (21) which were found
to be differentially expressed in senescent HUVECs were not present in current study. The
discrepancies found most likely due to HUVECs which originate from different donor and
different sensitivity of detection methods used.

In summary, the present study reveals that hsa-miR-299-3p plays a role in cellular
senescence in HUVECs. Its ability to delay premature senescence is mediated, at least in parts
through IGF1. Further investigations are necessary to confirm its role in IGF1 signaling and to
determine whether how hsa-miR-299p acts in tandem with other miRNAs identified by the
present experiments.

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Disclosures: None
References


**Figure legends**

**Figure 1.** Characterization of senescence in HUVECs. (A) morphological changes, (B) SA-ß-gal staining, (C) percentage of SA-ß-gal positive cells, (D) real time growth profiles, (E) cell cycle analysis, (F) measurement of relative telomere lengths in young cells and replicative senescent HUVECs. Y indicates young cells; RS indicates replicative senescent cells. Data are mean ± SEM; *** P <0.001, ** P <0.01, * P <0.05.

**Figure 2.** (A) Gene expression profile of young control versus RS cells. Fold change: negative values indicate down-regulation, positive values indicate up-regulation; fold change cut-off: 2.0; p-value computation by Student *t* test; Multiple testing correction: Benjamini Hochberg False Discovery Rate; Corrected p-value cut-off: 0.05. (B) Homology region between hsa-miR-299-3p and 3’ UTR of IGF1, SLFN13 and HDAC9 mRNA sequence. (C) Computational simulation of biological interactions of differentially expressed target genes of hsa-miR-299-3p in senescent HUVECs as revealed by GeneSpring GX version 11.5.1 Pathway Analysis. IGF-1, HDAC9 and SFLN13 genes are boxed in red.

**Figure 3.** RT-PCR validation of (A) miRNAs and (B) target genes of interest. (C) The expression of hsa-miR-299-3p following transfection with A299-3p and growth medium control. Y indicates young cells; RS indicates replicative senescent cells; A299-3p indicates inhibitor of hsa-miR-299-3p; C_A indicates control antisense oligonucleotide; a.u. indicates arbitrary units; Data are mean ± SEM; *** P <0.001, ** P <0.01, * P <0.05.
Figure 4. mRNA expression of IGF1 following the knockdown of hsa-miR-299-3p in (A) passage 25 and (B) passage 19 cells and (C) a combination of miRNA (simultaneous inhibition of hsa-miR-134, -299-3p and -338-3p) at a range of concentration. A299-3p indicates inhibitor of hsa-miR-299-3p; CA indicates control antisense oligonucleotide; Comb indicates combination of miRNA inhibitor; a.u. indicates arbitrary units; Data are mean ± SEM; *** P <0.001; ** P <0.01, * P <0.05.

Figure 5. (A) Senescence-associated β-galactosidase (SA-β-gal) staining, (B) percentage of SA-β-gal positive cells and (C) percentage of viable cells after inhibition of hsa-miR-299-3p at 31.25, 62.5, 125, 250 and 500 nM. A299-3p indicates inhibitor of hsa-miR-299-3p; CA indicates control antisense oligonucleotide; Data are mean ± SEM; *** P <0.001, ** P <0.01, * P <0.05.

Figure 6. Real time growth profiles of RS cells after inhibition of hsa-miR-299-3p at 31.25, 62.5, 125, 250 and 500 nM. A299-3p indicates inhibitor of hsa-miR-299-3p; CA indicates control antisense oligonucleotide; RS indicates replicative senescent cells; AUC indicates area under curve after transfection; a.u. indicates arbitrary unit. *** P <0.001, ** P <0.01, * P <0.05.

Figure 7. Real time growth profiles after inhibition of hsa-miR-299-3p at 31.25, 62.5, 125, 250 and 500 nM in H2O2-induced premature senescent cells. A299-3p indicates inhibitor of hsa-miR-299-3p; CA indicates control antisense oligonucleotide; RS indicates replicative senescent cells; AUC indicates area under curve after transfection; a.u. indicates arbitrary unit. *** P <0.001, ** P <0.01, * P <0.05.
Figure 8. Cell migration profile after inhibition of hsa-miR-299-3p at 31.25, 62.5, 125, 250 and 500nM. A299-3p indicates inhibitor of hsa-miR-299-3p; $C_A$ indicates control antisense oligonucleotide. AUC indicates area under curve from zeroth hour to eighth hour; a.u. indicates arbitrary unit; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.  


Table 1: miRNA expression profile of young control versus RS cells.

<table>
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<th>Systematic Name</th>
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Fold change cut-off: 2.0; p-value computation by Student t test; Multiple testing correction: Benjamini Hochberg False Discovery Rate; Corrected p-value cut-off: 0.05. * indicates less predominantly expressed miRNA (from the opposite arm of the precursor); FC Absolute indicates fold change absolute.
Figure 3

(A) Fold change (a.u.) for different miRNAs:
- hsa-miR-130b
- hsa-miR-18b
- hsa-miR-299-3p
- hsa-miR-338-3p
- hsa-miR-134
- hsa-miR-17
- hsa-miR-188-5p

(B) Fold change (a.u.) for genes:
- IGF1
- SLFN13
- HDAC9

(C) Fold change (a.u.) for concentration of A299-3p (nM):
Figure 5

(A) C_A and A299-3p at different concentrations (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM).

(B) SA-ß-gal staining at different concentrations of C_A and A299-3p (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM).

(C) MTT assay for % of viable cells at different concentrations of C_A and A299-3p (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM).
Figure 6

(A) 31.25nM of C_A
    31.25nM of A299-3p

(B) 62.5nM of C_A
    62.5nM of A299-3p

(C) 125nM of C_A
    125nM of A299-3p

(D) 250nM of C_A
    250nM of A299-3p

(E) 500nM of C_A
    500nM of A299-3p

Normalized cell index vs. Time (Hour)

AUC after transfection (a.u.)
Figure 7

(A) Normalized cell index over time for 31.25nM of CA and 31.25nM of A299-3p.

(B) Normalized cell index over time for 62.5nM of CA and 62.5nM of A299-3p.

(C) Normalized cell index over time for 125nM of CA and 125nM of A299-3p.

(D) AUC after transfection for 250nM of CA and 250nM of A299-3p.

(E) AUC after transfection for 500nM of CA and 500nM of A299-3p.

Different concentrations of CA and A299-3p are plotted over time, showing variations in normalized cell index and AUC after transfection.

(AUC after transfection (a.u.) values are marked with asterisks for statistical significance.)