MicroRNA 217 Modulates Endothelial Cell Senescence via Silent Information Regulator 1

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Background—Aging is a major risk factor for the development of atherosclerosis and coronary artery disease. Through a microarray approach, we have identified a microRNA (miR-217) that is progressively expressed in endothelial cells with aging. miR-217 regulates the expression of silent information regulator 1 (SirT1), a major regulator of longevity and metabolic disorders that is progressively reduced in multiple tissues during aging.

Methods and Results—miR-217 inhibits SirT1 expression through a miR-217–binding site within the 3′-UTR of SirT1. In young human umbilical vein endothelial cells, human aortic endothelial cells, and human coronary artery endothelial cells, miR-217 induces a premature senescence-like phenotype and leads to an impairment in angiogenesis via inhibition of SirT1 and modulation of FoxO1 (forkhead box O1) and endothelial nitric oxide synthase acetylation. Conversely, inhibition of miR-217 in old endothelial cells ultimately reduces senescence and increases angiogenic activity via an increase in SirT1. miR-217 is expressed in human atherosclerotic lesions and is negatively correlated with SirT1 expression and with FoxO1 acetylation status.

Conclusions—Our data pinpoint miR-217 as an endogenous inhibitor of SirT1, which promotes endothelial senescence and is potentially amenable to therapeutic manipulation for prevention of endothelial dysfunction in metabolic disorders. (Circulation. 2009;120:1524-1532.)

Key Words: endothelial cells ▪ aging ▪ atherosclerosis ▪ SirT1 protein ▪ microRNAs

The critical role of the endothelium in governing vascular, tissue homeostatic, and pathological processes is increasingly recognized.1 Cellular senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherogenesis,2 although the mechanisms underlying the aging-induced attenuation of endothelium-dependent functions are yet to be clarified.

Clinical Perspective on p 1532

SirT1 (silent information regulator 1) is an NAD+-dependent deacetylase that regulates gene expression by deacetylation of modified lysine residues on histones, transcription factors, and some transcription cofactors.3 Furthermore, SirT1 gain of function improves metabolic efficiency, promoting longevity and disease resistance.4–6 Consequently, SirT1 has been shown to exert protective effects against endothelial dysfunction by preventing stress-induced senescence7 and to play a key role in angiogenesis through the deacetylation of the forkhead transcription factor FoxO1.8 However, little is known about the mechanisms that regulate the decline in SirT1 during endothelial aging.7

MicroRNAs (miRNAs) are a class of endogenous, small, noncoding, single-stranded RNAs of approximately 22 nucleotides encoded within the genome and derived from endogenous short hairpin precursors. The mature miRNAs negatively regulate gene expression by targeting specific messenger RNAs (mRNAs) for cleavage or translational repression.9 A growing body of evidence suggests that they are involved in the control of a wide range of physiological pathways, such as development, differentiation, growth, and metabolism, as well as in disease conditions.10,11 Recent evidence has implicated overall miRNA levels in regulating angiogenesis and endothelial function.12–16 Here, we found that a particular miRNA, miR-217, is progressively expressed in endothelial cells during aging. In silico analysis indicates that SirT1 is a potential target of miR-217. Thus, in the present studies, we sought to address whether endothelial senescence is determined by a miR-217–dependent SirT1 loss of function.

Methods

Cells and Cell Culture

Human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAECs), and human coronary artery endothelial
cells (HCAECs) were purchased from Lonza (Basel, Switzerland) and cultured as described previously. Population-doubling levels (PDLs) were calculated as described previously; briefly, the number of population doublings (PD) that occurred between passages was calculated according to the equation \( PD = \log_2(Ch/Cs) \), where \( Ch \) is the number of viable cells at harvest and \( Cs \) is the number of cells seeded. All experiments were performed at the PDLs indicated in the text.

Isolation of miRNA and Microarray Analysis
Enriched miRNA was isolated from HUVECs with the mirVana miRNA Isolation Kit (Ambion Inc, Austin, Texas), which allows isolation of both enriched miRNA and larger RNA species separately. A MirVana miRNA Bioarray (Ambion) was used to compare miRNA expression in PDL8 and PDL44 cells. Four micrograms of enriched small RNA fractions was labeled fluorescently and hybridized to the bioarrays according to the manufacturer’s instructions. The slides were scanned on a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, Calif) at the optimal wavelength for the Alexa555 (F532) and Alexa647 (F635) dyes (Molecular Probes/Invitrogen, Carlsbad, Calif) with lasers. The acquired images were analyzed with GenePix Pro 5.0 (Molecular Devices). miRNA spots were automatically segmented, local backgrounds (B635 and B532) were subtracted, and both total intensities (F635 and F532) and the fluorescence ratios of the 2 dyes were calculated for each spot. The spots were flagged when they exhibited poor hybridization signals, when they were saturated (F635 or F532, median = 65 535), or when the signal-to-background ratio was <2. 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. GSE14912. Bioinformatic prediction of target genes and miRNA binding sites was performed with different miRNA databases, namely, miRBase (http://microrna.sanger.ac.uk/), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/index.html).

Northern Blot Analysis
A total of 1.5 \( \mu \)g of enriched small RNA fractions or 15 \( \mu \)g of total RNA was resolved on a 15% TBE-urea gel (Invitrogen) in 1X TBE buffer. Gels were stained briefly with ethidium bromide, and RNAs were transferred by electroblotting onto GeneScreen Plus membranes (PerkinElmer Life Sciences, Waltham, Mass) for 2 hours at 20 V in 0.5X TBE. The membranes were UV cross-linked (Stratalinker, Stratagene, La Jolla, Calif). Prehybridization was performed for 1 hour at 42°C in ULTRAhyb-Oligo hybridization buffer (Ambion), and the membranes were hybridized overnight at 42°C in the same buffer with the labeled probe. Antisense RNA probes were 3′-end-labeled with \( ^{32}P \)-y-ATP by T4 polynucleotide kinase; mir-217 antisense probe sequence TCCAATCAGTTCCTGATGCAGTA and U2 spliceosomal RNA antisense probe sequence GGTGCACCGTTCCTGGAGTAC were used. The membranes were washed twice for 30 minutes in 0.5% SDS, 2X SSC and exposed on an Imaging Screen-K (Bio-Rad, Hercules, Calif) at room temperature. Image scanning and analysis were performed on a personal molecular imager with QuantityOne software (Bio-Rad).

miRNA and miRNA Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis
Real-time quantification to measure miRNAs was performed with the TaqMan miRNA reverse transcription kit and miRNA assay according to the manufacturer’s protocol with the ABI PRISM 7000 system (Applied Biosystems, Foster City, Calif). The U43 small nuclear RNA (RNU43) was used as the housekeeping small RNA reference gene. For miRNA analysis, single-strand complementary DNA (cDNA) was synthesized from 1 \( \mu \)g of total RNA sample isolated through TRIzol reagent (Invitrogen) with a high-capacity cDNA archive kit according to the standard protocol. Fifty nanograms of cDNA was amplified by real-time polymerase chain reaction (RT-PCR) and normalized to 18S ribosomal RNA as an endogenous control. Each reaction was performed in triplicate, and analysis was performed by the 2\(^{-\Delta\Delta CT} \) method as described previously. Primer identification numbers (Applied Biosystems) were as follows: let7a, 4373169; hsa-mir-21, 4373090; hsa-mir-31, 4373190; hsa-mir-34, 4373278; hsa-mir-92, 4373013; hsa-mir-181b, 4373116; hsa-mir-216, 4373083; hsa-mir-217, 4373082; hsa-mir-503, 4373228; and RNU43, 4373375.

miRNA Transfection
Nucleofection of HUVECs and HAEcs was performed according to the manufacturer’s instructions with the Nucleofector machine (Amazaxa/Lonza). Cells (1×10\(^5\)) were resuspended in 100 \( \mu \)L of HUVEC nucleofection solution (Amazaxa/Lonza), and 0 to 40 nmol/L of mirIDIAN miR-217 mimic (miR217), 20 nmol/L of mirIDIAN miR-216 mimic (miR216), 20 nmol/L of mirIDIAN mir-217 inhibitor (A217), or 0 to 40 nmol/L of control scramble sequences (respectively, Co and C\(_2\), Dharmacon, Lafayette, Colo) was added. Samples were transferred into certified cuvettes (Amazaxa/Lonza) and transfected with program A-034. Nucleofection of HCAECs was performed in HCAEC nucleofector solution (Amazaxa/Lonza) with program S-005. Fresh medium (500 \( \mu \)L) was added immediately after transfection to each cuvette, and the cells were plated and incubated at 37°C for 3 days.

Luciferase Assay
The SirT1 3′-UTR sequence spanning 1940 base pairs (bp) was PCR amplified with primers (+) TGA TGG AGA TGA TCA AGA AGC and (−) GAC CCA TGA TGA TCA AGA GGC. This region contains the mir-217 consensus response element, located between 3740 and 3769 bp downstream of the ATG of sequence AF083106. The amplicon was inserted into the XhoI site of the pGCL3 control vector (Promega, Madison, Wis) downstream of the cDNA for the luciferase reporter gene. Empty pGCL3-basic and pGCL3-SirT1-3′-UTR were used to test mir-217 activity. Each vector, along with varying doses (20 and 40 nmol/L) of miR217, was transfected into human embryonic kidney 293 (HEK293) cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were cultured for 2 days and assayed with the Dual-Luciferase Reporter Assay System (Promega).

In Vitro Angiogenesis Assay
For tube formation assays, HUVECs were transfected, and after 18 hours, cells (1×10\(^5\)) were seeded on a 12-well plate coated with 200 \( \mu \)L of ECMatrix Solution according to the standard protocol (Chemicon International, Temecula, Calif). Tube length was quantified after 24 hours by measuring the cumulative tube length in 5 random microscopic fields with a computer-assisted microscope using the Lucia program, as described previously.

Telomerase Assay
HUVECs were washed in PBS and lysed, and telomerase activity was measured by the TeloTAGGG Telomerase PCR ELISA kit (Roche Molecular Biochemicals, Indianapolis, Ind).

Senescence-Associated β-Galactosidase Staining
At 10 days after transfection, HUVECs and HAEcs were fixed, and the senescent status was verified by in situ staining for senescence-associated β-galactosidase (SA-β-gal) as described previously. Briefly, cells were grown on 6-well culture plates, washed with PBS, and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes. After another washing step with PBS, cells were incubated with β-galactosidase staining solution (150 mmol/L NaCl, 2 mmol/L MgCl\(_2\), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 40 mmol/L citric acid, 12 mmol/L sodium phosphate, pH 6.0, containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-gal]) for 24 hours at 37°C. The reaction was stopped by the addition of PBS. Statistical analysis was performed by counting at least 600 cells for each condition. As a positive control, PDL8 cells transfected with control oligonucleotide were treated.
with 100 μmol/L H₂O₂ at 37°C for 1 hour to generate oxidative stress, which leads to cellular senescence.

Western Blot Analysis and Immunoprecipitation
Western blots were performed as described previously with the following antibodies: Anti-eNOS (BD Transduction Laboratories, San Jose, Calif), anti-SirT1 (Abcam Inc, Cambridge, Mass), anti-FoxO1 and anti-acetyl-FoxO1 K242 (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-acetyl-lysine (Cell Signaling), anti-acetylated p53 (lys382) and p53 (Cell Signaling Technology, Danvers, Mass), anti-actin (Santa Cruz Biotechnology), and anti-tubulin (Sigma, St Louis, Mo). Immunoprecipitation experiments were performed as described previously with 1 to 2 mg of total cell extracts for each sample. Where indicated, cells were treated with nicotinamide 5 mmol/L for 3 hours before lysis.

Atherosclerotic Plaque Sampling
Frozen human atherosclerotic plaque samples (n=63) from patients who underwent carotid endarterectomy for symptomatic disease were homogenized with a polytron homogenizer in Trizol reagents (Invitrogen) for total RNA isolation and in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% Tween 20, 1 μg/mL aprotinin, 1 mmol/L PMSF) for cell protein lysates. Real-time PCR and Western blot analysis were performed as described above.

Statistical Analysis
Group differences of continuous variables were compared by 1-way ANOVA or Student t test as appropriate. Relationships between variables were determined by the Pearson correlation coefficient. Continuous data are expressed as mean±SD. A P value <0.05 was considered statistically significant. All analyses were performed with GraphPad Prism 5.0 (GraphPad, San Diego, Calif) and SPSS software program version 13.0 (SPSS Inc, Chicago, Ill) for Windows.

Results

miR-217 Expression Is Upregulated in Aging Endothelial Cells
To investigate the role of miRNAs in endothelial cell senescence, we first profiled the miRNA signature during HUVEC aging using a DNA microarray for mature forms of miRNAs of human, mouse, and rat origin. Analysis of samples from PDL8 HUVECs revealed that the present results were in keeping with previous reports from other groups. In particular, the most highly expressed miRNAs in our baseline (miR-126, miR-16, miR-21, miR-221, and miR-222) overlapped with those from published databases. Next, we compared the miRNA profile obtained in old HUVECs (PDL44) versus young cells (PDL8; online-only Data Supplement Figure I). We found several miRNAs were significantly upregulated with aging (Figure 1A); among these, miR-217 (located on chromosome 2p16.1) was the most significantly differentially expressed miRNA. To validate the present findings, we analyzed the most significantly changed miRNAs by RT-PCR, which confirmed the results obtained by microarray, with the exception of miR-92 and miR-503 (Figure 1B).

SirT1 Is a Potential Target of miR-217
Computational miRNA target analysis from miRNA databases identified homology between miR-217 and a region that extended between 3740 and 3769 bp from the ATG (GB extended between 3740 and 3769 bp from the ATG (GB 216-8). We confirmed this gene as a potential molecular target for miR-217 (Figure 2A), which is also induced with aging (Figure 1), was not linked to SirT1 by computational analysis; by contrast, miR-34 was demonstrated to affect SirT1. However, their variation in both microarray analysis and RT-PCR was less significant than that of miR-217 (online-only Data Supplement Figure I; Figure 1A and 1B). Western blot analysis (Figure 2F and 2G).

miR-217 Downregulates Endogenous SirT1 Expression in HUVECs and HAECS
To further confirm the hypothesis that miR-217 negatively regulates SirT1 expression, we preliminarily transfected PDL8 HUVECs with different concentrations of miR217 or AF083106) in the 3′-UTR of the human SirT1 RNA, which establishes this gene as a potential molecular target for miR-217 (Figure 2A), miR-216, which is also induced with aging (Figure 1), was not linked to SirT1 by computational analysis; by contrast, miR-34 was demonstrated to affect SirT1. However, their variation in both microarray analysis and RT-PCR was less significant than that of miR-217 (online-only Data Supplement Figure I; Figure 1A and 1B). Northern blot assays and quantitative RT-PCR analysis with primers that recognize mature miR-217, which mimics mature miR-217, with SirT1 3′-UTR reporter in HEK293 cells resulted in 40% inhibition of luciferase activity compared with samples transfected with the control oligonucleotide (Figure 2H).

AF083106) in the 3′-UTR of the human SirT1 RNA, which establishes this gene as a potential molecular target for miR-217 (Figure 2A), miR-216, which is also induced with aging (Figure 1), was not linked to SirT1 by computational analysis; by contrast, miR-34 was demonstrated to affect SirT1. However, their variation in both microarray analysis and RT-PCR was less significant than that of miR-217 (online-only Data Supplement Figure I; Figure 1A and 1B). Northern blot assays and quantitative RT-PCR analysis with primers that recognize mature miR-217, which mimics mature miR-217, with SirT1 3′-UTR reporter in HEK293 cells resulted in 40% inhibition of luciferase activity compared with samples transfected with the control oligonucleotide (Figure 2H).
control oligonucleotide. After 3 days, both RT-PCR (n=3) and immunoblotting (n=2) revealed that SirT1 levels were significantly diminished in PDL8 HUVECs transfected with miR217 compared with controls, with slightly increasing efficacy ranging from 5 to 40 nmol/L (online-only Data Supplement Figure IIA and IIB). On the basis of these results, we chose a final concentration of 20 nmol/L (Figure 3A and 3B). Next, we analyzed PDL44 HUVECs, in which we observed that the reduction of miR-217 levels by transfection with the same amount of antisense oligonucleotide (A217) led to an increase in SirT1 expression after 3 days. Moreover, in PDL44 HUVECs, cotransfection with A217 and miR217 reversed the increase of SirT1 expression, reducing the RNA and protein levels above control values (Figure 3C and 3D). After transfection, the miR-217 levels were determined by RT-PCR or Northern blot (online-only Data Supplement Figures IIC through IIF). To establish the relevance of the present findings to other endothelium models, we analyzed the effect of miR-217 in PDL8 HAECs. Both RT-PCR and immunoblotting revealed that SirT1 levels were significantly diminished in PDL8 cells transfected with miR217 compared with controls (Figure 3E).

miR-217 Induces a Premature Senescent-Like Phenotype and Regulates Angiogenic Activity in HUVECs

Because SirT1 inhibition is associated with premature senescence in HUVECs, we tested the role of miR-217 in HUVEC senescence. First, we examined the effect of modulation of miR-217 levels on SA-β-gal activity, a characteristic feature of senescence-related growth arrest. In PDL8 HUVECs, we found that miR217 overexpression increased the percentage of SA-β-gal–positive cells by 30% compared with control at 10 days after transfection. Moreover, miR217 increased SA-β-gal–positive cells by 15% compared with the cellular senescence positive control (100 μmol/L H2O2; Figure 4A and 4B). In PDL44 HUVECs, inhibition of miR-217 function by A217 resulted in a

Figure 2. miR-217 and SirT1 expression is coregulated during endothelial aging. A. The sequence of miR-217 and its potential matching site in the SirT1 3’-UTR. B and C, Northern blot (n=4). For positive control, we loaded 1 fmol of a miR-217 probe (TACTGCACTAG-GAACTGATTGGATT) (D) and E, RT-PCR (n=8) of miR-217 levels in small enriched RNA fractions and total RNA in HUVEC aging model. F and G, SirT1 mRNA and protein levels in HUVEC aging model; n=4 for each experiment. **P<0.01, ***P<0.001 by Student t test. H, miR217 dose-dependent suppression of luciferase activity in HEK293 cells transfected with pGL3-SirT1-3’UTR containing mir-217 consensus binding sequence in the SirT1-3’UTR; n=4 for each experiment. *P<0.05, **P<0.005, ***P<0.0005 by 1-way ANOVA. Data are mean±SEM. a.u. indicates arbitrary units; U2snRNA, U2 spliceosomal RNA; 5S-rRNA, 5S ribosomal RNA; and tub, tubulin.

Figure 3. miR-217 suppresses SirT1 expression. A and B, SirT1 mRNA and protein expression in PDL8 HUVECs transfected with miR217 (A and B) and in PDL44 HUVECs transfected with either A217 or with miR217 and A217 (C and D). E, SirT1 mRNA and protein expression in PDL8 HAECs transfected with miR-217 (n=4 for each experiment). *P<0.05, **P<0.005, ***P<0.0005 by Student t test or 1-way ANOVA (Figure 3D). Data are mean±SEM. Tub indicates tubulin; a.u., arbitrary units; Co, control oligonucleotide; and Cn, control antisense oligonucleotide.
significant decrease of SA-β-gal–positive cells (57% of A217 transfected cells compared with 88% of control). Cotransfection with miR217 significantly abrogated the effect of A217 on SA-β-gal activity (Figure 4A and 4B). Endothelial senescence is accompanied by telomerase inactivation.27 We found that in both PDL8 and PDL44 HUVECs, telomerase activity was dependent on modulation of miR-217 levels (Figure 4C), which confirms a crucial role for miR-217 in HUVEC senescence. Moreover, analysis of SA-β-gal activity in HAECs at PDL8 revealed a similar significant effect of miR217 (Figure 4D).

To further verify that this was a miR-217–specific activity, we analyzed the effect of miR216 on SA-β-gal activity, because miR-216 was similarly overexpressed in our preliminary microarray experiment. We found that miR216 was not able to reduce SirT1 expression (online-only Data Supplement Figure IIIA) or to induce senescence when injected alone, and it did not increase the miR217 effect on SA-β-gal activity, which suggests that miR-216 may possibly be involved in other aspects of the aging process (online-only Data Supplement Figure IIIB). To analyze the effects of miR-217 on vascular-like network formation, we performed an in vitro Matrigel assay. Transfection with miR217 in PDL8 HUVECs led to a significant impairment of tube-forming activity (Figure 5A); conversely, in PDL44 HUVECs, reduction of miR-217 with A217 resulted in rescued tube formation in the Matrigel assay (Figure 5B).

miR-217 Modulates SirT1 Deacetylase Activity in Endothelial Cells

To test whether the processes controlled by miR-217 could be mediated by a deacetylation-dependent regulation of SirT1
target genes, we first analyzed FoxO1 acetylation with an anti-FoxO1 acetylated lysine–specific antibody. In PDL8 HUVECs, overexpression of miR217 resulted in marked FoxO1 acetylation, similar to control cells treated with nicotinamide, an inhibitor of SirT1 deacetylase activity (Figure 6A). Interestingly, although we found that HUVEC senescence was associated with increased FoxO1 acetylation, we observed that it was not associated with increased p53 acetylation, and miR217 did not affect p53 acetylation, which suggests that it may act specifically to modulate angiogenic rather than apoptotic SirT1 targets (online-only Data Supplement Figure IVA and IVB). By contrast, in PDL44 HUVECs, A217 reduced FoxO1 acetylation levels (Figure 6B).

To confirm that FoxO1 is an indirect target of miR-217, we compared mRNA expression of genes coordinately regulated with SirT1 and FoxO1, similar to control cells treated with nicotinamide, an inhibitor of SirT1 deacetylase activity (Figure 6A). Interestingly, although we found that HUVEC senescence was associated with increased FoxO1 acetylation, we observed that it was not associated with increased p53 acetylation, and miR217 did not affect p53 acetylation, which suggests that it may act specifically to modulate angiogenic rather than apoptotic SirT1 targets (online-only Data Supplement Figure IVA and IVB). By contrast, in PDL44 HUVECs, A217 reduced FoxO1 acetylation levels (Figure 6B).

To confirm that FoxO1 is an indirect target of miR-217, we compared mRNA expression of genes coordinately regulated with SirT1 and FoxO1 in both young and old HUVECs transfected with miR217 or A217. The results showed that miR-217 is involved in the mRNA modulation of FoxO1 targets, such as the stress-resistance gene GADD45 (growth arrest and DNA-damage-inducible gene); Fms-related tyrosine kinase 1 (Flt-1), a crucial regulatory tyrosine kinase receptor for angiogenesis; and endothelial nitric oxide synthase (eNOS), which is essential for endothelial function (Figure 6C and 6D). Similar results on FoxO1 acetylation (Figure 6E) and FoxO1 gene targets (Figure 6F) were observed in HAECs. Gene-expression studies revealed that miR217 was also able to downregulate SirT1 and FoxO1 targets in HCAECs (online-only Data Supplement Figure VA).

Previously, it has been shown that SirT1 directly modulates eNOS expression and acetylation.28 We observed that miR-217 significantly affects eNOS levels in both PDL8 and PDL44 HUVECs (Figure 7A and 7B) and in HAECs (Figure 7C). Next, we investigated whether modulation of miR-217 affects eNOS acetylation in PDL8 cells. To avoid artifacts due to the different eNOS levels caused by miR217 overexpression and consequent effects on FoxO1 downregulation of eNOS,29 we performed immunoprecipitation assays using 2 mg of lysate, an amount of protein that exceeded the precipitating antibody capacity; in fact, immunoprecipitation of eNOS with 1 mg of protein did not allow for clear detection of the acetylation state (data not shown). The present results revealed that miR217 enhances eNOS acetylation when overexpressed in young cells. Conversely, A217 decreased eNOS acetylation in PDL44 HUVECs, an effect reversed by the presence of miR217 (Figure 7D and 7E), thus confirming that miR-217 is a regulator of the SirT1-eNOS axis.

MiR-217 Is Negatively Correlated With SirT1 Expression in Human Atherosclerotic Plaques

Functional changes in senescent vascular cells in vivo may play an important role in the pathophysiology of age-associated vascular disorders.3 Therefore, to investigate the function of miR-217 in vivo, we examined miR-217, SirT1, and SirT1 target expression in plaques from patients who underwent carotid endarterectomy for symptomatic disease (online-only Data Supplement Table I). The present results
confirmed a negative correlation between miR-217 and SirT1 mRNA level in an in vivo setting (Figure 8A). This result was further confirmed by the evidence that SirT1 correlated in a positive manner with its vascular targets GADD45, Flt1, and eNOS (Figure 8B through 8D). Next, we analyzed the atherosclerotic plaques that had the highest versus lowest miR-217 levels (n = 7 for each group; Figure 8E). These samples showed reciprocal levels for SirT1 mRNA and protein (Figure 8F through 8H). Moreover, Western blot assays confirmed that a reciprocal association exists in vivo between the expression levels of miR-217 and the acetylation state of FoxO1 (Figure 8G and 8I).

**Discussion**

Aging is one of the major risk factors for type 2 diabetes mellitus and atherosclerosis, the leading causes of morbidity and mortality in Western countries. Among the age-associated functional and structural changes, of particular note is the decline in endothelial function, the molecular basis of which is not fully defined.2 Endothelial growth and senescence are characterized by a progressive decrease in SirT1-dependent control of the FoxO1 transcription factor.8 SirT1 belongs to the class III histone deacetylases that have been implicated in the regulation of cellular senescence and longevity in several organisms and as master regulators of metabolic efficiency under different nutrient availability.

Loss of function of SirT1 and other sirtuins is unexplained. Here, we have shown that endothelial senescence is dependent on the age-progressive increase in miRNA miR-217, which affects SirT1 expression, leading to loss of SirT1 function on its major endothelial targets, FoxO1 and eNOS.28–31

**Figure 7.** Effect of miR-217 on eNOS expression and acetylation in endothelial cells. Regulation of eNOS protein levels (A and B) and eNOS acetylation (D and E) in PDL8 HUVECs transfected with miR217 and PDL44 HUVECs transfected either with A217 or with miR217 and A217, respectively. Cells treated with the SirT1 inhibitor nicotinamide (NAM, 5 mmol/L) for 3 hours served as a positive acetylation control. C, eNOS protein levels in PDL8 HAECs transfected with miR217. n = 4 for each experiment. *P < 0.05, **P < 0.005 by Student t test or 1-way ANOVA. Data are mean ± SEM. Co indicates control oligonucleotide; CA, control antisense oligonucleotide; tub, tubulin; a.u., arbitrary units; IP, immunoprecipitation; and WB, Western blot.

**Figure 8.** miR-217 and SirT1 expression in vivo. A, Correlation between SirT1 and miR-217 expression in human atherosclerotic plaques. B, C, and D, Correlation between SirT1 and eNOS, Flt1, and GADD45 expression in human atherosclerotic plaques; n = 63. E and F, MiR-217 and SirT1 levels in plaques with high miR-217 levels (n = 7) or low miR-217 levels (n = 7). G, H, and I, SirT1 protein expression and FoxO1 acetylation in plaques with high or low miR-217 levels. ***P < 0.0005 by Student t test. Data are mean ± SEM. a.u. indicates arbitrary units.
The present data are consistent with a role of miR-217 as the natural inhibitor of SirT1 during endothelial senescence. First, miR-217 expression is absent in young cells and progressively emerges during repeated rounds of cellular replication. This is paralleled by a decrease in SirT1 expression. Second, modulation of miR-217 is able to induce cellular senescence in young cells, whereas inhibition of miR-217 rescues, in part, aging phenotypes in old cells. This is reflected by the impact of miR-217 on tube formation in a Matrigel assay, which is rescued once miR-217 levels are reduced by its synthetic antagonist. Third, miR-217 affects the acetylation state of 2 major targets of SirT1 in endothelial function, FoxO1 and eNOS, which are both determinant factors for angiogenesis and tube formation.

In particular, FoxO1 acts as a negative regulator of angiogenesis and vascular growth, and it is hypothesized to also act as a sensor of cellular metabolism and health at chromatin genesis and vascular growth, and it is hypothesized to also act as a sensor of cellular metabolism and health at chromatin

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18. Menini S, Amoruso R, Fiorentino L, Casagrande V, Menghini R, Menini S, Amoruso R, Fiorentino L, Casagrande V, Menghini R, Lauro R. miR-217 expression is absent in young cells and progressively emerges during repeated rounds of cellular replication. This is paralleled by a decrease in SirT1 expression. Second, modulation of miR-217 is able to induce cellular senescence in young cells, whereas inhibition of miR-217 rescues, in part, aging phenotypes in old cells. This is reflected by the impact of miR-217 on tube formation in a Matrigel assay, which is rescued once miR-217 levels are reduced by its synthetic antagonist. Third, miR-217 affects the acetylation state of 2 major targets of SirT1 in endothelial function, FoxO1 and eNOS, which are both determinant factors for angiogenesis and tube formation.

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Disclosures

None.


**CLINICAL PERSPECTIVE**

Cellular senescence of endothelial cells has been involved in causes of atherosclerosis, although the mechanisms underlying the aging-induced attenuation of endothelial functions are unknown. On the basis of recent evidence, we hypothesized that microRNAs, a class of endogenous, small, noncoding, single-stranded RNAs of approximately 22 nucleotides that are known to negatively regulate gene expression, may be a cause of endothelial dysfunction. In the present study, we used a model of endothelial senescence to identify microRNAs associated with the aging process and to recognize their potential targets. We found that a particular microRNA, *miR-217*, is progressively expressed in endothelial cells during senescence. In silico analysis indicated that silent information regulator 1 (SirT1) is a potential target of *miR-217*. SirT1 is an NAD⁺-dependent deacetylase that regulates gene expression and exerts protective effects against endothelial dysfunction and metabolic syndrome. SirT1 action is lost during aging, but the cause of the age-related decline of SirT1 is unknown. Thus, in the present studies, we sought to address whether endothelial senescence is determined by a *miR-217*-dependent SirT1 loss of function. We observed that *miR-217* inhibits SirT1 function in endothelial cells, specifically by reducing its ability to modulate the function of a transcription factor called FoxO1 that is involved in angiogenesis, apoptosis, and stress resistance. The inhibition of *miR-217* rescues the phenotype of senescent endothelial cells. The evidence for such a *miR-217*-SirT1-FoxO1 pathway in atherosclerotic plaque from human donors has clear clinical implications for the possibility of interfering with *miR-217* by use of treatments to antagonize its function in the endothelium of patients.
MicroRNA 217 Modulates Endothelial Cell Senescence via Silent Information Regulator 1
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Supplementary Figure 1. **MicroRNA expression profile in human endothelial cells during aging**

Small RNA fractions were purified from PDLs8-HUVEC or PDLs44-HUVEC, labeled with a fluorescent dye and hybridized to a microarray chip. Expression data were normalized to the level of the control miRNA (n=2 for each experiment with a intra-assay duplicate).

Supplementary Figure 2. **SirT1 is a target of miR-217**

(A and B) Dose-dependent suppression of SirT1 mRNA and protein levels by miR217 in PDLs8-transfected HUVEC. (C) RT-PCR of miR-217 in PDLs8-HUVEC transfected with 20nM miR217 and (D and E) RT-PCR and (F) Northern Blot in PDLs44-HUVEC transfected with either 20nM A217, or with 20nM miR217 and 20nM A217, respectively. (n=4 for each experiment). **p<0.005, ***p<0.001 by Student’s T test or one-way ANOVA. Data are mean ± SEM.

Supplementary Figure 3. **SirT1 is not a target of miR-216**

(A) SirT1 mRNA expression and (B) Photomicrographs of SA-β–gal staining and SA-β-gal positive cells in PDLs8-HUVEC transfected with miR216 and/or miR217. (n=3 for each experiment); **p<0.005, ***p<0.0005 by One-way ANOVA. Data are mean ± SEM.

Supplementary Figure 4. **p53 acetylation in HUVEC aging model**

(A) p53 and FoxO1 acetylation levels in the HUVEC aging model. (B) p53 acetylation level in PDLs8-HUVEC transfected with miR217. Cells treated with the SirT1 inhibitor nicotinamide (NAM, 5 mM) for 3 hours served as a positive acetylation control (n=3 for each experiment).

Supplementary Figure 5. **MiR217 modulates SirT1 and FoxO1 targets in HCAEC**

(A) SirT1 and FoxO1 target genes expression in PDLs8-HCAEC transfected with miR217; (n=4 for each experiment). *p< 0.05, ***p<0.0005 by one-way ANOVA. Data are mean ± SEM.
A

PDLs
8 44 8+NAM

Acetyl-Lys p53
p53
Acetyl-Lys FoxO1
FoxO1

B

Co Co + NAM miR217

Acetyl-Lys p53
p53

PDLs8
A

HCAEC PDLs8

Supplementary 5
**miR-217 modulates endothelial cell senescence via SirT1**

Rossella Menghini,¹ et al

Supplemental Table 1. Clinical characteristics of patients who underwent carotid endarterectomy

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<tr>
<td>N.</td>
<td>63</td>
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<tr>
<td>Men/women</td>
<td>37/26</td>
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<tr>
<td>Smoking status (Yes/No)</td>
<td>17/46</td>
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<tr>
<td>Age (yrs)</td>
<td>70.8 ± 6.25</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>26.11 ± 3.74</td>
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<tr>
<td>Waist (cm)</td>
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<tr>
<td>Systolic Blood Pressure (mmHg)</td>
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<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
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<tr>
<td>Total Cholesterol (mg/dl)</td>
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<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td>45.42 ± 11.16</td>
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<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td>120.65 ± 36.24</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>144.28 ± 73.71</td>
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<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>104.53 ± 14.23</td>
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<td>2h-post load plasma glucose (mg/dl)</td>
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<td>Fasting plasma insulin (uU/ml)</td>
<td>11.26 ± 7.26</td>
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<td>2h-post load plasma insulin (uU/ml)</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.65 ± 0.47</td>
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<td>HOMA IR</td>
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<tr>
<td>NGT/IGT/T2DM</td>
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<tr>
<td>hsCRP (mg/l)</td>
<td>6.03 ± 16.55</td>
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HOMA IR, Homeostasis Model Assessment for Insulin Resistance
NGT, Normal Glucose Tolerant
IGT, Impaired Glucose Tolerant
T2DM, Type 2 Diabetes Mellitus
hsCRP, high sensitive C Reactive Protein