Journals of Gerontology: Biological SCIENCES, 2014, 1–8
doi:10.1093/gerona/glu180
Original Article

Original Article

MicroRNA-34a Induces Vascular Smooth Muscle Cells Senescence by SIRT1 Downregulation and Promotes the Expression of Age-Associated Pro-inflammatory Secretory Factors

Ileana Badi,[1,2] Ilaria Burba,[1,3] Clarissa Ruggeri,[1,2] Filippo Zeni,[1,2] Matteo Bertolotti,[1,2] Alessandro Scopece,[1] Giulio Pompilio,[1] and Angela Raucci[1,2]

[1]Unit of Vascular Biology and Regenerative Medicine and [2]Unit of Cardiovascular Regeneration and Aging, Centro Cardiologico Monzino-IRCCS, Milan, Italy.

[3]Present address: Department of Translational Medicine and Surgery, University of Milano-Bicocca, Milan, Italy.

Address correspondence to Angela Raucci, PhD, Unit of Cardiovascular Regeneration and Aging, Centro Cardiologico Monzino, Via C. Parea 4, 20138 Milan, Italy. Email: araucci@ccfm.it

Abstract

Arterial aging is a major risk factor for the occurrence of cardiovascular diseases. The aged artery is characterized by endothelial dysfunction and vascular smooth muscle cells altered physiology together with low-grade chronic inflammation. MicroRNA-34a (miR-34a) has been recently implicated in cardiac, endothelial, and endothelial progenitor cell senescence; however, its contribution to aging-associated vascular smooth muscle cells phenotype has not been explored so far. We found that miR-34a was highly expressed in aortas isolated from old mice. Moreover, its well-known target, the longevity-associated protein SIRT1, was significantly downregulated during aging in both endothelial cells and vascular smooth muscle cells. Increased miR-34a as well as decreased SIRT1 expression was also observed in replicative-senescent human aortic smooth muscle cells. miR-34a overexpression in proliferative human aortic smooth muscle cells caused cell cycle arrest along with enhanced p21 protein levels and evidence of cell senescence. Furthermore, miR-34a ectopic expression induced pro-inflammatory senescence-associated secretory phenotype molecules. Finally, SIRT1 protein significantly decreased upon miR-34a overexpression and restoration of its levels rescued miR-34a-dependent human aortic smooth muscle cells senescence, but not senescence-associated secretory phenotype factors upregulation. Taken together, our findings suggest that aging-associated increase of miR-34a expression levels, by promoting vascular smooth muscle cells senescence and inflammation through SIRT1 downregulation and senescence-associated secretory phenotype factors induction, respectively, may lead to arterial dysfunctions.

Key Words: MiR-34a—SIRT1—Vascular aging—SASP—Inflammation.

Decision Editor: Rafael de Cabo, PhD

Age-associated detrimental changes of arterial anatomy and physiology are important risk factors for cardiovascular morbidity and mortality (1). Aged arteries are characterized by endothelial dysfunction and a heterogeneous phenotypic shift of vascular smooth muscle

cells (VSMCs) that affects their contractility, migration, extracellular matrix synthesis and deposition, proliferation, and senescence. Interestingly, the molecular mechanisms that lead to these changes in the aged vessels are also important for the pathogenesis of vascular diseases (VDs) such as atherosclerosis and hypertension (2).

Cellular senescence is a hallmark of organismal aging characterized by irreversible growth arrest and altered cell morphology, accumulation of DNA damage/oxidative stress, telomere shortening, mitochondrial dysfunction and increased expression of senescence-associated β-galactosidase (SA-β-gal), cyclin-dependent kinase inhibitors p21 and p16, and the tumor suppressor gene p53 (3,4). Furthermore, cells approaching senescence develop a senescence-associated secretory phenotype (SASP) consisting in the secretion of pro-inflammatory molecules such as cytokines, chemokines, proteases, growth factors, and soluble receptors that acting in a paracrine and/or autocrine manner modulates tissue microenvironment (3–5). SASP factors secreted by senescent VSMCs contribute to the maintenance of a chronic low-grade inflammation typical of aged vasculature (2,6). Identification of new molecular players that influence the mechanisms regulating intrinsic vascular aging and related dysfunctions is of high scientific interest.

MicroRNAs (miRNAs) are small, noncoding RNAs that control gene expression by targeting messenger RNAs (7) that are emerging as new therapeutic targets. Indeed, miRNAs deregulation profoundly affects tissues development and homeostasis, and is involved in the onset of various diseases; moreover, the expression and function of these small molecules can be efficiently manipulated in vivo through systemic or local delivery of miRNA inhibitors or mimics (8,9). MicroRNA-34a (miR-34a) is a p53-regulated tumor suppressor miRNA, able to control cell cycle arrest, apoptosis, and senescence in a p53-dependent or -independent and cell cancer context manner (10–12). More recently, several studies have highlighted miR-34a as an important mediator of aging and cardiac and endothelial cell dysfunctions (13,14). miR-34a expression increases in different organs of aged mice such as brain, spleen, and heart (14–17). In the heart, genetic ablation of miR-34a mitigates contractile function decline and prevents cardiac hypertrophy of old mice reducing cardiomyocytes apoptosis (14). Further, miR-34a expression rises in the border zone of the infarcted heart and its inhibition improves remodeling after infarction (14). Finally, increasing levels of miR-34a are observed during endothelial and endothelial progenitor cells culturing and are responsible for the acquisition of their senescent phenotype through direct downregulation of one of its target genes, *SIRT1* (15,17).

This study was undertaken to test the hypothesis that miR-34a could impact vascular aging and, in particular, VSMCs senescence. We demonstrated that miR-34a expression increases in aged mouse aorta and replicative-senescent primary human aortic smooth muscle cells (HASMCs) along with a reduction of SIRT1 protein levels. Overexpression of miR-34a in proliferative HASMCs induces G0–G1 cell cycle arrest, SIRT1-dependent senescence, and contributes to their inflammatory phenotype by triggering the expression of age-associated pro-inflammatory molecules. Our data suggest that miR-34a by influencing VSMCs senescence and inflammation may play a key role in regulating age-associated arterial diseases.

Methods

An expanded Methods section is provided in the online Supplementary Material.

Animal work was performed in accordance with national and international law and policies.

Results

miR-34a Expression Increases During Aging in Murine Aortas

To investigate whether miR-34a expression is modulated during the process of vascular aging, we analyzed aortas isolated from young (2.5-month-old) and old (21-month-old) mice. As expected and revealed by Western blot (WB) analysis, compared with young specimens, aged aortas showed a significant increase in the levels of p16 (Supplementary Figure 1A and B) and p21 (Supplementary Figure 1C and D), two senescence-associated proteins. The expression levels of SM22a, a contractile smooth muscle cells marker, were also significantly downregulated in the old vasculature (Supplementary Figure 1E). Notably, as determined by quantitative reverse transcriptase–PCR, we observed a nearly twofold increase of miR-34a expression levels in aged aortas compared with young aortas (Figure 1A).

SIRT1 Expression Decreases With Aging in Both Endothelial and VSMCs

We, then, assessed the protein levels of Axl, Bcl-2, and SIRT1 by WB analyses; these are known miR-34a targets and play a role in vascular cell function (18–23). Neither Axl nor Bcl-2 protein levels exhibited a significant difference in young versus old aortas (Supplementary Figure 2A–D). Conversely, SIRT1 protein levels were significantly downregulated with age (Supplementary Figure 2E and F). Immunohistochemical analysis of aortic sections showed SIRT1 expression in both endothelial and VSMCs in young aortas (Figure 1B). Interestingly, a strong reduction of SIRT1 expression was observed in both endothelial and VSMCs of aged aortas (Figure 1B and C). The quantification of the immuno-positive areas in young versus old aortic sections confirmed the results obtained with the WB analysis (Supplementary Figure 2E and F). The decrease of SIRT1 expression in aged aortas correlated with the increased percentage of nuclei positively stained for the senescence marker p16 (Supplementary Figure 3A and B).

miR-34a Expression Increases During Replicative Senescence in HASMCs Along With a Reduction of SIRT1 Protein Levels

We next determined if miR-34a expression is modulated during VSMCs aging. As replicative senescence is considered an underlying cause of aging (24), we cultured HASMCs until they ceased to proliferate (passage 15). HASMCs at passage 5 were used as a young control. Senescence at passage 15 was confirmed by SA-β-gal staining (Supplementary Figure 4) and, in accordance to previous studies, senescent cells exhibited increased gene expression of several pro-inflammatory molecules (data not shown) (6,25). Real-time PCR revealed a robust increase of miR-34a expression levels in senescent versus young cells (Figure 1D). Conversely, WB analysis indicated a strong downregulation of SIRT1 protein levels in old HASMCs compared with young cells (Figure 1E). Similar results were observed in two other HASMCs clones (data not shown).

miR-34a Overexpression Inhibits HASMCs Proliferation and Induces Cell Senescence

To investigate whether the upregulation of miR-34a associated to HASMCs senescence plays a role in VSMCs growth, we assessed the effects of miR-34a ectopic expression on young HASMCs proliferation by transfecting these cells with a miR-34a mimic. In comparison with the control (miRNA mimic control [SCR]), miR-34a

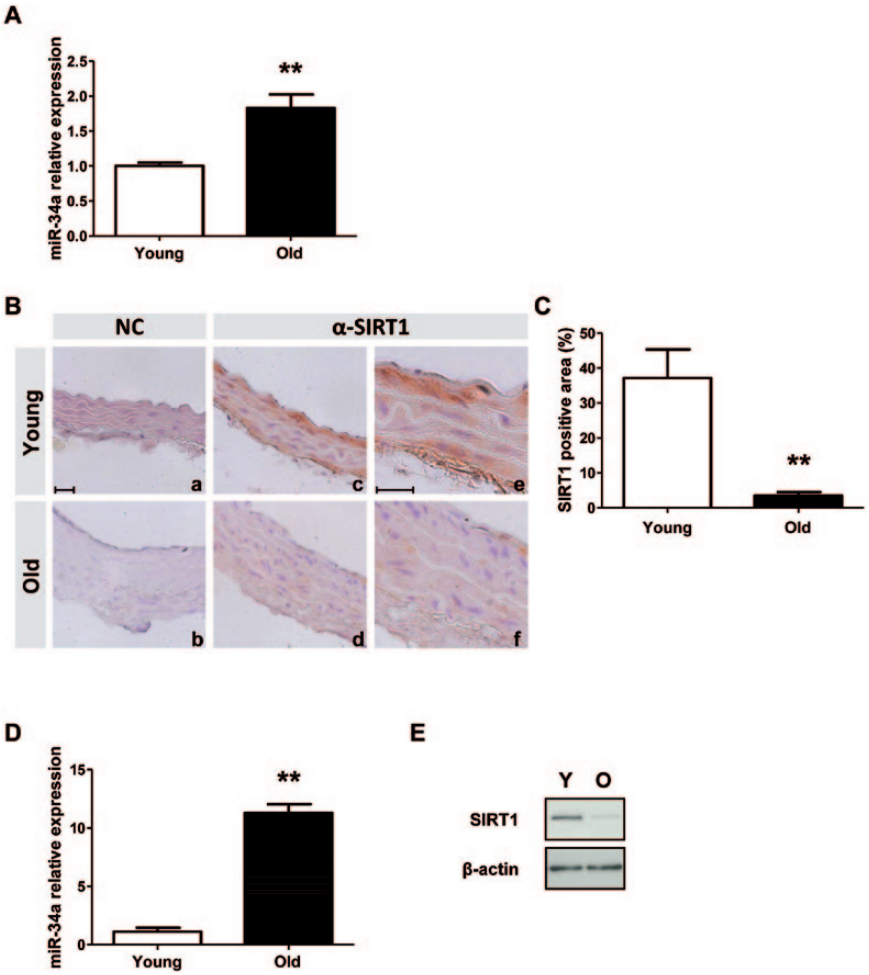

Figure 1. MicroRNA-34a (miR-34a) is upregulated and SIRT1 is downregulated in aged mouse aortas and in senescent human aortic smooth muscle cells (HASMCs). (**A**) miR-34a expression in murine aortas was analyzed by quantitative reverse transcriptase–PCR (q-RT–PCR) and normalized to corresponding snoRNA202 levels, (n = 4,7). (**B**) Representative images of aortic sections from young and old mice stained for SIRT1 (brown) or nothing (negative control, NC). Nuclei were counterstained with hematoxylin (purple). Original magnification images (×200) are shown in a, b, c, and d, whereas high-magnification images (×400) are shown in e and f; scale bar: 20 μm. (**C**) Bars show the percentage of SIRT1-positively stained area to the total aortic area, (n = 5). (**D**) miR-34a expression in HASMCs was analyzed by q-RT–PCR and normalized to corresponding RNU6B levels. (**E**) WB showing SIRT1 or β-actin (loading control) levels in young and senescent HASMCs (Y: young, early passage; O: old, late passage).Values are mean ± standard error. $^{**}p < .01$.

mimic reduced cell number already by 48 hours after transfection; the main and statistically significant differences were observed at 72-hours post-transfection (Figure 2A). Further, we evaluated cell cycle distribution by fluorescence-activated cell sorting analysis after propidium iodide (PI) staining. miR-34a ectopic expression significantly increased the percentage of HASMCs in G0–G1 phase (Figure 2B) at 24-hours post-transfection when compared with negative control. Accordingly, p21 protein levels were higher in miR-34a-overexpressing cells (Figure 2C and D) (26).

We, then, evaluated whether miR-34a could modulate cell senescence, by performing a SA-β-gal staining at different time points (24, 48, and 72 hours) on HASMCs transfected either with miR-34a mimic or SCR. As a control, we cultured along proliferative (young) and senescent (old) cells. We found that miR-34a enhanced the percentage of SA-β-gal-positive cells at 72 hours after transfection (miR-34a vs SCR; Figure 2E), while HASMCs transfected with the negative control did not show any difference when compared with untransfected cells (SCR vs young; Figure 2E).

We also performed SA-β-gal staining at different time points on HASMCs transfected either with a miR-34a inhibitor or a negative control and we observed that miR-34a inhibition could prevent senescence at 72 hours after transfection (Figure 2F).

These results suggest that miR-34a overexpression inhibits VSMCs proliferation by inducing G0–G1 cell cycle arrest and promotes senescence.

miR-34a Overexpression Stimulates the Induction of Pro-inflammatory Factors in HASMCs

We next tested whether miR-34a overexpression in young HASMCs induced the inflammatory phenotype of senescent VSMCs (data not shown) (6,25). At 72 hours after transfection, compared with SCR, the miR-34a significantly increased the messenger RNA levels of pro-inflammatory cytokines IL1β, IL8, IL6, and BMP2 and the chemokine MCP1, as well as the soluble adhesion molecule ICAM1 (Table 1). Interestingly, IL6, MCP1, and ICAM were already upregulated by 48-hours post-transfection likely in pre-senescent cells (Table 1). On the contrary, miR-34a did not influence gene expression of growth factors or related proteins (Table 1).

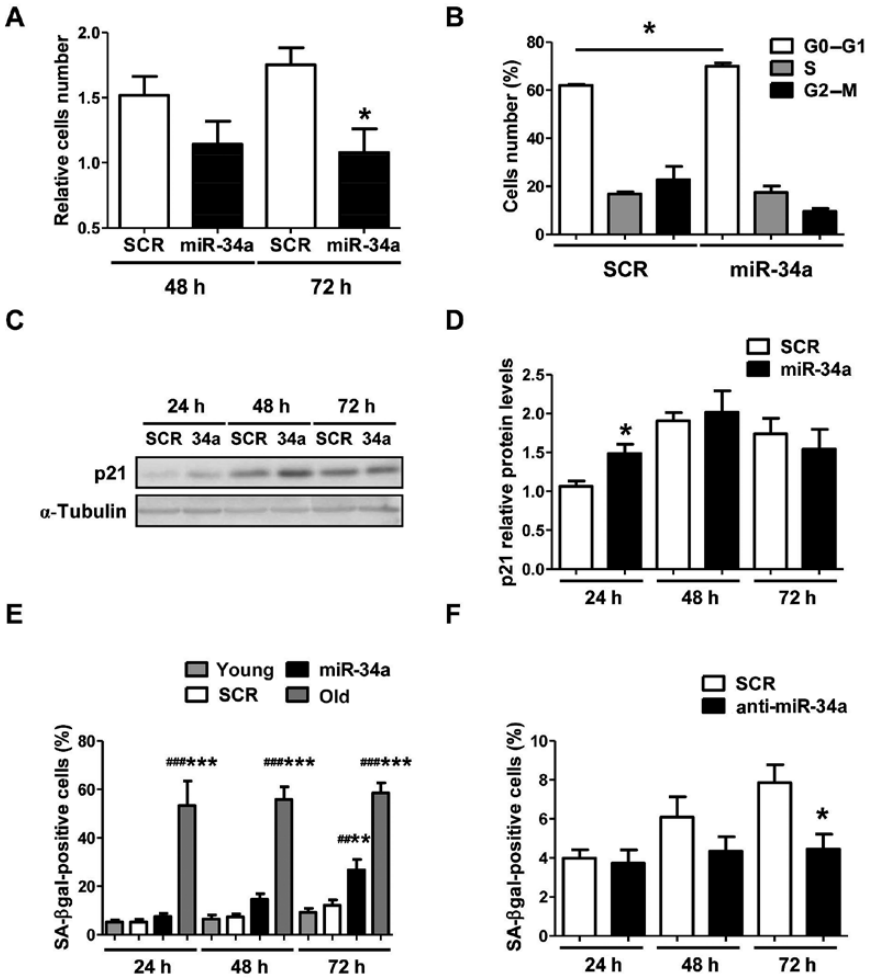

Figure 2. MicroRNA-34a (miR-34a) overexpression in young human aortic smooth muscle cells (HASMCs) inhibits proliferation and induces senescence. (**A–E**) Cells were transfected with a miRNA mimic control (SCR) or a miR-34a mimic (miR-34a) and processed at the indicated times. (A) Cells were counted 48 and 72 hours after transfection. Data represent fold change versus the number of plated cells, ($n = 3$). (B) Flow cytometric analysis with propidium iodide staining of cells 24 hours after transfections. Bars show cell cycle phase distribution percentages, ($n = 3$). (C) Cell extracts were subjected to WB with anti-p21 or anti-α-tubulin (loading control) antibody. One representative experiment is shown. (D) Bars show normalized densitometric ratios, ($n = 3$). (E) Bars represent the percentages of senescence-associated β-galactosidase (SA-β-gal)-positive cells; (young: early passage; old: late passage), $n \geq 3$. (**F**) Cells were transfected with a miR-34a hairpin inhibitor (anti-miR-34a) or a hairpin inhibitor negative control (SCR), cultured for 24, 48, and 72 hours, and stained for SA-β-gal; ($n = 5$). Values are mean ± standard error. *$p < .05$; **$p < .01$; ***$p < .0001$ vs SCR; ##$p < .01$; ###$p < .0001$ vs young.

These results indicate that increasing levels of miR-34a contribute to the inflammatory phenotype of aged VSMCs.

miR-34a Can Induce VSMCs Senescence Through the Direct Modulation of SIRT1

As SIRT1 has been shown to influence VSMCs proliferation and senescence (27), we explored the possibility that miR-34a could impact both processes by directly regulating SIRT1 protein levels. We detected a decrease of SIRT1 protein expression in HASMCs that ectopically expressed miR-34a at 48 hours but not 24 or 72 hours after transfection in comparison with control (Figure 3A and B). This indicates that miR-34a-dependent SIRT1 downregulation occurs after HASMCs G0–G1 cell cycle arrest and anticipates senescence induced by high expression of miR-34a.

We then performed a rescue experiment by transfecting replicative cells with miR-34a mimic or mimic negative control along with either a 3′-untraslated region-deleted-SIRT1-expression vector (devoid of the sequences matching miR-34a seed sequence) or the corresponding empty vector. The WB analysis confirmed that endogenous SIRT1 protein levels were severely lowered upon miR-34a overexpression, while exogenous SIRT1 protein levels were unaffected (Figure 3C). We next assessed cell proliferation and senescence 72 hours after transfection. As expected, miR-34a inhibited cell proliferation and increased senescence compared with mimic control (empty SCR vs empty miR-34a; Figure 3D and E). SIRT1 ectopic expression alone did not affect the cell number and senescence compared with negative control (empty SCR vs SIRT1 SCR; Figure 3D and E) and, in combination with miR-34a, could not inhibit miR-34a-dependent growth arrest but partially reversed the miR-34a-induced senescence (empty miR-34a vs SIRT1 miR-34a; Figure 3D and E).

We also performed the rescue experiment in the presence of H_2O_2, known to induce premature senescence, and evaluated cell growth and the percentage of SA-β-gal-positive cells 24 hours after transfection. In these conditions, miR-34a inhibited cell proliferation and increased senescence compared with mimic control (empty SCR vs empty miR-34a; Figure 3F and G). SIRT1 ectopic expression

Table 1. SASP Factors Relative Expression Upon miR-34a Overexpression in HASMCs

Gene	Fold Δ in Expression (48 h/*t* = 0)			Fold Δ in Expression (72 h/*t* = 0)		
	SCR	miR-34a	*p*	SCR	miR-34a	*p*
BMP2	0.73 ± 0.11	0.70 ± 0.10	.81	0.36 ± 0.06	0.64 ± 0.07	.04*
GRO/α	2.82 ± 0.57	3.16 ± 0.70	.74	4.84 ± 1.49	6.57 ± 0.64	.29
ICAM1	4.01 ± 0.56	10.19 ± 1.81	.04*	17.15 ± 2.13	51.46 ± 9.00	.02*
IGFBP4	0.61 ± 0.12	0.76 ± 0.04	.26	0.74 ± 0.06	1.45 ± 0.47	.26
IGFBP6	0.57 ± 0.12	0.44 ± 0.09	.43	0.46 ± 0.06	0.44 ± 0.18	.93
IL1β	1.80 ± 0.57	3.39 ± 1.01	.27	0.84 ± 0.07	2.78 ± 0.50	.02*
IL6	1.41 ± 0.25	4.94 ± 0.78	.01*	6.25 ± 1.32	18.41 ± 2.72	.02*
IL8	0.44 ± 0.11	0.95 ± 0.30	.21	0.32 ± 0.13	0.76 ± 0.08	.03*
MCP1	1.21 ± 0.34	6.26 ± 1.05	.01*	1.78 ± 0.33	8.69 ± 2.28	.05*
OPG	0.91 ± 0.34	0.57 ± 0.21	.40	1.08 ± 0.45	0.72 ± 0.20	.44
VEGF	0.64 ± 0.10	0.97 ± 0.12	.10	0.49 ± 0.03	0.67 ± 0.08	.14

Notes: BMP2 = bone morphogenetic protein 2; GRO/α = growth-related oncogene/α; HASMC = human aortic smooth muscle cells; ICAM1 = intercellular adhesion molecule 1; IGFBP4/6 = insulin-like growth factor binding protein 4/6; IL1β/6/8 = interleukin 1 β/6/8; MCP1 = monocyte chemoattractant protein 1; miR-34a = microRNA-34a; OPG = osteoprotegerin; SASP = senescence-associated secretory phenotype; SCR = miRNA mimic control; VEGF = vascular endothelial growth factor.

Data are fold change in mean ± standard error of messenger RNA expression in SCR- or miR-34a-transfected HASMCs at different time points compared with cells at time 0.

*$p < .05$ vs SCR.

alone induced a slight but not statistically significant increase in cell number compared with negative control (empty SCR vs SIRT1 SCR; Figure 3F). Combination of SIRT1 and miR-34a was associated with a trend toward inhibition of cell proliferation versus SIRT1 SCR, which did not achieve statistical significance (Figure 3F). Further, SIRT1 overexpression had no effect on cell senescence compared with negative control (empty SCR vs SIRT1 SCR; Figure 3G), but it was able to counteract the effect of miR-34a (empty miR-34a vs SIRT1 miR-34a; Figure 3G).

All together these data indicate that miR-34a regulates VSMCs senescence, but not cell proliferation, through the direct modulation of SIRT1.

miR-34a-Mediated Upregulation of Pro-inflammatory SASP Factors Is SIRT1 Independent

As SIRT1 knockdown in adipocytes induces the expression IL1β, MCP1, and IL6 and its activation by resveratrol reduces the secretion of these molecules by VSMCs (28,29), we checked whether SIRT1 was able to reverse the miR-34a-mediated induction of pro-inflammatory SASP factors expression in the absence or presence of H_2O_2. As expected, miR-34a was able to increase the expression levels of the assessed molecules (empty SCR vs empty miR-34a; Supplementary Figure 5). The presence of SIRT1 could not counteract the miR-34a effect (empty miR-34a vs SIRT1 miR-34a; Supplementary Figure 5).

Hence, miR-34a-mediated transcriptional activation of pro-inflammatory SASP factors is SIRT1 independent.

Discussion

Cellular senescence contributes to vascular aging as well as to the development and progression of age-related VDs (30). Several miRNAs appear to be deregulated during cell senescence and are likely to play a role in age-dependent VD (13). In this study, we have investigated the unexplored role of miR-34a in vascular aging and, specifically, in VSMCs senescence.

miR-34a has been already associated with endothelial senescence, and its expression has been shown to increase with age in different human and murine tissues (14–17); however, to our knowledge, this is the first evidence reporting an upregulation of this miRNA in aged arteries (Figure 1A). Accordingly, protein levels of two senescence-related proteins, p21 and p16, increased as well in old vessels, whereas the expression of SM22a significantly decreased (Supplementary Figure 1). SM22a plays an active role in maintaining the differentiated contractile phenotype of VSMCs and exerts atheroprotective effects as its genetic ablation in mice has been shown to augment the atherosclerotic lesion area (31,32). Notably, among all the assessed miR-34a targets, only SIRT1 protein levels were significantly downregulated during aging (Supplementary Figure 2); indeed, we observed high SIRT1 expression in young arterial endothelial cells and VSMCs, whereas it was barely detectable in both cell types of aged vessels (Figure 1B). Although Axl and Bcl-2 have been described as miR-34a targets (18,21), their protein levels did not change significantly between young and old aortas, suggesting that their expression is likely affected by other regulatory mechanisms during aging; moreover, miR-34a modulation of these genes may be cell specific.

In accordance to the in vivo data, miR-34a levels increase, whereas SIRT1 protein expression decreases, in replicative-senescent VSMCs in vitro (Figure 1D and E). Furthermore, ectopic induction of miR-34a in proliferative HASMCs causes at first, cell cycle arrest in G0–G1 phase, along with an expected p21 upregulation, and later, an augmentation of cell senescence (Figure 2). Thus, as it occurs for endothelial cells (15), miR-34a regulates VSMCs proliferation and senescence, and it is plausible that modulation of its expression contributes to intrinsic vascular aging and/or VD.

SIRT1 is a negative regulator of cell senescence (33) and has been shown to mitigate several aging-associated degenerative and metabolic diseases (34). Although the beneficial effects of SIRT1 on endothelial cells behavior in VD have been extensively studied, its role and regulation in VSMCs function in age-dependent arterial dysfunction are still relatively unknown (27,35). Recently, Gorenne and *colleagues* (27) demonstrated that the VSMCs-specific ablation of *Sirt1* gene in the ApoE−/− background promotes atherogenesis and enhances medial degeneration. Moreover, SIRT1 is downregulated in VSMCs derived from human atherosclerotic plaques (27) and retards senescence-dependent VSMCs calcification in vitro (36). Thus, SIRT1 can counteract VSMCs senescence-mediated processes of atherogenesis and vascular calcification (VC) (30,37). In this study, we showed that miR-34a upregulation in VSMCs significantly reduces SIRT1 protein levels (Figure 3A and B) and miR-34a-mediated VSMCs replicative senescence and H_2O_2-induced premature senescence occur through modulation of SIRT1 (Figure 3E and G). Indeed, although stress-induced premature senescence and replicative senescence differ in the triggering mechanisms, they share some of the main effectors and both are involved in VD (3,30). Notably, in absence of H_2O_2, SIRT1 could only partially counteract the miR-34a-induced senescence (Figure 3E vs Figure 3G) suggesting a different extent of involvement. In contrast, miR-34a-induced inhibition of VSMCs proliferation is SIRT1 independent (Figure 3D and F) because the G0–G1 cell growth arrest and p21 upregulation precede SIRT1 downregulation. Additional experiments are needed to establish whether miR-34a could be a pivotal player in VSMCs senescence-mediated vascular dysfunction through SIRT1 control.

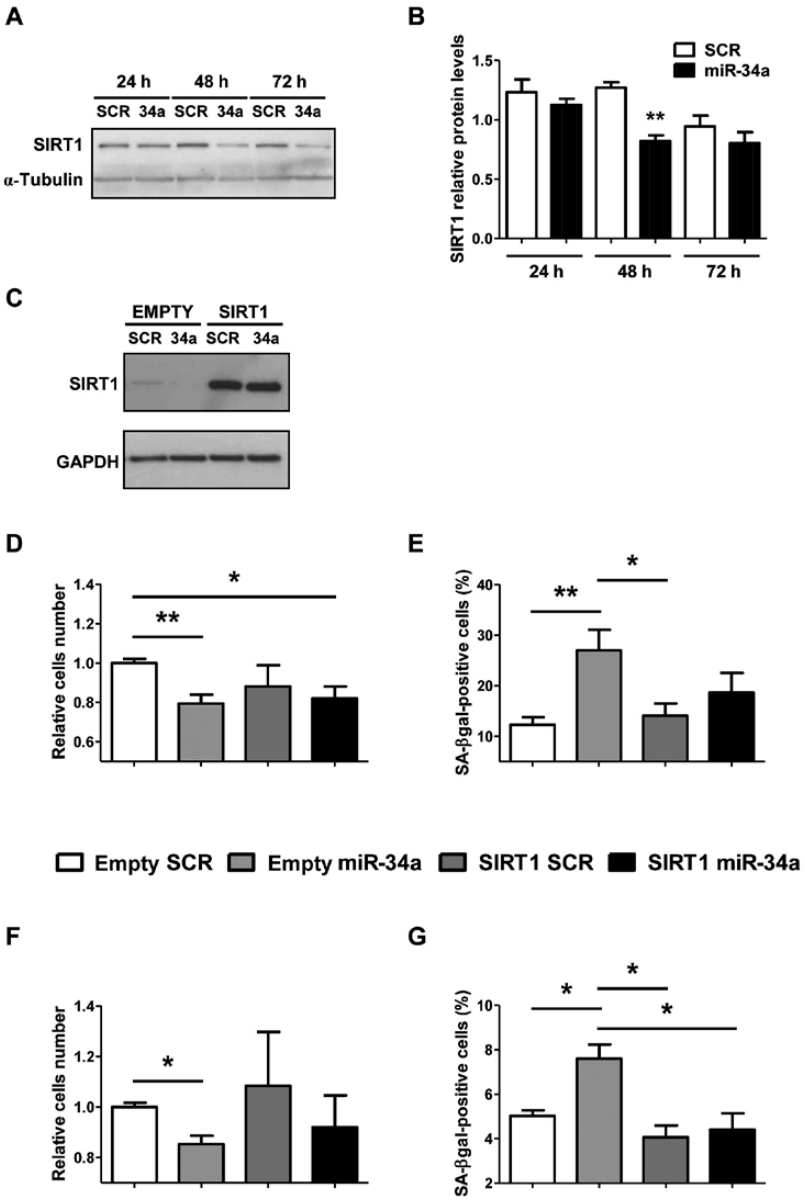

Figure 3. MicroRNA-34a (miR-34a) can induce vascular smooth muscle cells senescence through SIRT1 modulation. (A and B) Cells were transfected with a miR-34a mimic (miR-34a) or a mimic control (SCR) and cultured for 24, 48, and 72 hours. (A) Cell extracts were subjected to WB with anti-SIRT1 or anti-α-tubulin (loading control) antibody. (B) Bars show normalized densitometric ratios, ($n = 4$). (C–G) HASMCs were transfected with miR-34a (34a) or SCR along with either a 3′-untraslated region-deleted-SIRT1-expression vector (SIRT1) or an empty vector (EMPTY). (C) Cells were cultured for 24 hours, and cell extracts were subjected to WB with anti-SIRT1 or anti-GAPDH (loading control) antibody. (D and E) Cells were cultured for 72 hours and cell number (D) or percentages of senescence-associated β-galactosidase (SA-β-gal)-positive cells (E) were determined. (F and G) Cells were cultured in presence of H_2O_2 for 24 hours and cell number (F) or percentages of SA-β-gal-positive cells (G) were determined; ($n \geq 3$). Values are mean ± standard error. *$p < .05$; **$p < .01$.

Surprisingly, in our in vitro assays, the overexpression of SIRT1 per se, failed to either reduce senescence or enhance VSMCs proliferation (Figure 3D–G); this may depend on our experimental conditions as well as on the expression levels of the exogenous protein. Accordingly, conflicting results have been reported in the literature. Li and *colleagues* described an inhibitory effect of SIRT1 overexpression on VSMCs proliferation both in vitro and in vivo, whereas Ho and *colleagues* showed that SIRT1 promotes VSMCs proliferation by extending their replicative life span (38,39). Besides, both SIRT1 overexpression and VSMCs-specific ablation of *Sirt1* gene in mice exacerbate atherogenesis (27,40). Therefore, enhancing SIRT1 expression beyond certain levels could be a detrimental therapeutic strategy to cure age-associated VD, whereas restoring SIRT1 physiological expression may be an effective approach. As miRNAs are emerging as a new potential class of drug targets, a therapeutically useful modality could be based on chemistries that inhibit miRNAs which, in turn, target SIRT1 (41). One possible candidate may be miR-34a.

Arterial aging is also characterized by low-grade chronic inflammation due, in part, to the acquisition of the pro-inflammatory phenotype by senescent VSMCs that start secreting SASP factors (data not shown) (6,29). Notably, and for the first time, we showed that overexpression of miR-34a enables HASMCs to express some SASP molecules, specifically, inflammatory cytokines,

and chemokines such as IL1β, IL6, IL8, MCP1, BMP2, and the soluble adhesion receptor ICAM1 (Table 1). Conversely, growth factors and their regulators (ie, IGFBP4/6, GRO/α, and VEGF), known to be implicated in tissue remodeling (42,43), were not modulated (Table 1). Of note, the expression kinetic of SASP factors is differentially influenced by miR-34a, in particular, levels of IL6, MCP1, and ICAM1 (the most induced ones) increase earlier compared with BMP2, IL8, or IL1β (Table 1). This is in agreement with data reporting secretion of SASP molecules by pre-senescent VSMCs (44). It is plausible that miR-34a stimulates these "early" cytokines to drive the induction of "late" SASP factors in order to reinforce VSMCs senescence (45).

Taking in consideration that the acquisition of pro-inflammatory phenotype by pre-senescent/senescent VSMCs has been correlated to the development of age-associated VD such as atherosclerosis and VC, it is even more likely an involvement of miR-34a in the onset of these vascular dysfunctions (6,25,44). Indeed, IL6, the most abundant pro-inflammatory cytokine secreted by aged VSMCs, IL8 and BMP2 are implicated in both atherosclerosis and VC (6,46). MCP1 and ICAM1 may be secreted by VSMCs to generate a pro-inflammatory environment and promote recruitment of inflammatory cells, thus enhancing the atherosclerotic process (30). IL1β can regulate VSMCs switch from a contractile to an inflammatory phenotype by either repressing the transcription of SM22a, or inducing the expression of other SASP factors, including ICAM1 and MCP1 (47–49). Interestingly, osteoprotegerin expression is not affected by miR-34a overexpression and, even if it has been found upregulated in senescent VSMCs (25), recent data suggest a protective role on atherosclerosis and VC (50,51). Finally, aortas from ApoE–/– mice fed a Western diet displayed an increase of miR-34a expression during the progression of atherosclerosis (52), and in agreement with the animal studies, miR-34a was found upregulated in human atherosclerotic plaques of a small cohort of patients (53).

Interestingly, the miR-34a-mediated upregulation of SASP molecules is SIRT1 independent (Supplementary Figure 5). The molecular mechanisms by which this miRNA induces SASP factors production may be various. It has been reported that secretion of IL6 and MCP1 in aged human VSMCs depends on TLR4/Myd88 expression and activation (6). Secretion of IL1β, MCP1, TNFα, and IL6 in aged *Macaca mulatta* VSMCs correlates with increased NF-kB activation (29). Mounting evidences also demonstrate that some miRNAs can act as agonist of TLRs and, through the activation of NF-kB, eventually trigger a pro-inflammatory response (54–56). Determining whether miR-34a regulates TLR4/MyD88 and/or NF-kB expression or activation by acting as a direct TLRs agonist or by targeting negative regulators of these pathways is of extreme importance in order to develop an effective strategy to counteract miR-34a potential impact on vascular aging and related complications.

In conclusions, our findings suggest a model in which aging-associated increase of miR-34a expression levels in VSMCs could lead to SIRT1 downregulation and SASP factors induction, whereby exacerbating cell senescence and inflammation that characterize arterial dysfunctions such as VC and atherosclerosis (Supplementary Figure 6).

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

Funding

This work was supported by Fondazione Monzino and Centro Cardiologico Monzino-IRCCS (RC2012-2015); Fondazione Umberto Veronesi (2013 to G.P.).

Acknowledgments

We thank Stefania Di Maggio for providing oligos and Fondazione Umberto Veronesi for the financial support.

Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the author(s).

References

1. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. *Circulation*. 2003;107:139–146. doi:10.1161/01.CIR.0000048892.83521.58
2. Wang M, Jiang L, Monticone RE, Lakatta EG. Proinflammation: the key to arterial aging. *Trends Endocrinol Metab*. 2014;25:72–79. doi:10.1016/j.tem.2013.10.002
3. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev*. 2010;24:2463–2479. doi:10.1101/gad.1971610
4. Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. *Genes Dev*. 2014;28:99–114. doi:10.1101/gad.235184.113
5. Campisi J. Cellular senescence: putting the paradoxes in perspective. *Curr Opin Genet Dev*. 2011;21:107–112. doi:10.1016/j.gde.2010.10.005
6. Song Y, Shen H, Schenten D, Shan P, Lee PJ, Goldstein DR. Aging enhances the basal production of IL-6 and CCL2 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2012;32:103–109. doi:10.1161/ATVBAHA.111.236349
7. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136:215–233. doi:10.1016/j.cell.2009.01.002
8. Thum T. MicroRNA therapeutics in cardiovascular medicine. *EMBO Mol Med*. 2012;4:3–14. doi:10.1002/emmm.201100191
9. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov*. 2012;11:860–872. doi:10.1038/nrd3864
10. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ*. 2010;17:193–199. doi:10.1038/cdd.2009.56
11. Concepcion CP, Han YC, Mu P, et al.et al. Intact p53-dependent responses in miR-34-deficient mice. *PLoS Genet*. 2012;8:e1002797. doi:10.1371/journal.pgen.1002797
12. Okada N, Lin CP, Ribeiro MC, et al.et al. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. *Genes Dev*. 2014;28:438–450. doi:10.1101/gad.233585.113
13. Dimmeler S, Nicotera P. MicroRNAs in age-related diseases. *EMBO Mol Med*. 2013;5:180–190. doi:10.1002/emmm.201201986
14. Boon RA, Iekushi K, Lechner S, et al.et al. MicroRNA-34a regulates cardiac ageing and function. *Nature*. 2013;495:107–110. doi:10.1038/nature11919
15. Ito T, Yagi S, Yamakuchi M. MicroRNA-34a regulation of endothelial senescence. *Biochem Biophys Res Commun*. 2010;398:735–740. doi:10.1016/j.bbrc.2010.07.012
16. Li X, Khanna A, Li N, Wang E. Circulatory miR34a as an RNAbased, noninvasive biomarker for brain aging. *Aging (Albany NY)*. 2011;3:985–1002.
17. Xu Q, Seeger FH, Castillo J, et al.et al. Micro-RNA-34a contributes to the impaired function of bone marrow-derived mononuclear cells from patients with cardiovascular disease. *J Am Coll Cardiol*. 2012;59:2107–2117. doi:10.1016/j.jacc.2012.02.033
18. Mudduluru G, Ceppi P, Kumarswamy R, Scagliotti GV, Papotti M, Allgayer H. Regulation of Axl receptor tyrosine kinase expression by miR-

34a and miR-199a/b in solid cancer. *Oncogene*. 2011;30:2888–2899. doi:10.1038/onc.2011.13
19. Melaragno MG, Fridell YW, Berk BC. The Gas6/Axl system: a novel regulator of vascular cell function. *Trends Cardiovasc Med*. 1999;9:250–253. doi:10.1016/S1050-1738(00)00027-X
20. Nishio E, Watanabe Y. Oxysterols induced apoptosis in cultured smooth muscle cells through CPP32 protease activation and bcl-2 protein downregulation. *Biochem Biophys Res Commun*. 1996;226:928–934. doi:10.1006/bbrc.1996.1452
21. Chang TC, Wentzel EA, Kent OA, et al.et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell*. 2007;26:745–752. doi:10.1016/j.molcel.2007.05.010
22. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci USA*. 2008;105:13421–13426. doi:10.1073/pnas.0801613105
23. Stein S, Matter CM. Protective roles of SIRT1 in atherosclerosis. *Cell Cycle*. 2011;10:640–647. doi:10.4161/cc.10.4.14863
24. Dimri GP, Lee X, Basile G, et al.et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA*. 1995;92:9363–9367.
25. Burton DG, Giles PJ, Sheerin AN, et al.et al. Microarray analysis of senescent vascular smooth muscle cells: a link to atherosclerosis and vascular calcification. *Exp Gerontol*. 2009;44:659–665. doi:10.1016/j.exger.2009.07.004
26. Tanner FC, Boehm M, Akyürek LM, et al.et al. Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. *Circulation*. 2000;101:2022–2025. doi:10.1161/01.CIR.101.17.2022
27. Gorenne I, Kumar S, Gray K, et al.et al. Vascular smooth muscle cell sirtuin 1 protects against DNA damage and inhibits atherosclerosis. *Circulation*. 2013;127:386–396. doi:10.1161/CIRCULATIONAHA.112.124404
28. Yoshizaki T, Milne JC, Imamura T, et al.et al. SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes. *Mol Cell Biol*. 2009;29:1363–1374. doi:10.1128/MCB.00705-08
29. Csiszar A, Sosnowska D, Wang M, Lakatta EG, Sonntag WE, Ungvari Z. Age-associated proinflammatory secretory phenotype in vascular smooth muscle cells from the non-human primate *Macaca mulatta*: reversal by resveratrol treatment. *J Gerontol A Biol Sci Med Sci*. 2012;67:811–820. doi:10.1093/gerona/glr228
30. Wang JC, Bennett M. Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circ Res*. 2012;111:245–259. doi:10.1161/CIRCRESAHA
31. Feil S, Hofmann F, Feil R. SM22alpha modulates vascular smooth muscle cell phenotype during atherogenesis. *Circ Res*. 2004;94:863–865. doi:10.1161/01.RES.0000126417.38728.F6
32. Han M, Dong LH, Zheng B, Shi JH, Wen JK, Cheng Y. Smooth muscle 22 alpha maintains the differentiated phenotype of vascular smooth muscle cells by inducing filamentous actin bundling. *Life Sci*. 2009;84:394–401. doi:10.1016/j.lfs.2008.11.017
33. Yao H, Chung S, Hwang JW, et al.et al. SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. *J Clin Invest*. 2012;122:2032–2045. doi:10.1172/JCI60132
34. Yamamoto H, Schoonjans K, Auwerx J. Sirtuin functions in health and disease. *Mol Endocrinol*. 2007;21:1745–1755. doi:10.1210/me.2007-0079
35. Borradaile NM, Pickering JG. NAD(+), sirtuins, and cardiovascular disease. *Curr Pharm Des*. 2009;15:110–117. doi:10.2174/138161209787185742
36. Takemura A, Iijima K, Ota H, et al.et al. Sirtuin 1 retards hyperphosphatemia-induced calcification of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2011;31:2054–2062. doi:10.1161/ATVBAHA.110.216739
37. Nakano-Kurimoto R, Ikeda K, Uraoka M, et al.et al. Replicative senescence of vascular smooth muscle cells enhances the calcification through initiating the osteoblastic transition. *Am J Physiol Heart Circ Physiol*. 2009;297:H1673–H1684. doi:10.1152/ajpheart.00455.2009
38. Ho C, van der Veer E, Akawi O, Pickering JG. SIRT1 markedly extends replicative lifespan if the NAD+ salvage pathway is enhanced. *FEBS Lett*. 2009;583:3081–3085. doi:10.1016/j.febslet.2009.08.031
39. Li L, Zhang HN, Chen HZ, et al.et al. SIRT1 acts as a modulator of neointima formation following vascular injury in mice. *Circ Res*. 2011;108:1180–1189. doi:10.1161/CIRCRESAHA.110.237875
40. Qiang L, Lin HV, Kim-Muller JY, Welch CL, Gu W, Accili D. Proatherogenic abnormalities of lipid metabolism in SirT1 transgenic mice are mediated through Creb deacetylation. *Cell Metab*. 2011;14:758–767. doi:10.1016/j.cmet.2011.10.007
41. van Rooij E, Purcell AL, Levin AA. Developing microRNA therapeutics. *Circ Res*. 2012;110:496–507. doi:10.1161/CIRCRESAHA.111.247916
42. Breland UM, Halvorsen B, Hol J, et al.et al. A potential role of the CXC chemokine GROalpha in atherosclerosis and plaque destabilization: downregulatory effects of statins. *Arterioscler Thromb Vasc Biol*. 2008;28:1005–1011. doi:10.1161/ATVBAHA.108.162305
43. Xie L, Tsaprailis G, Chen QM. Proteomic identification of insulin-like growth factor-binding protein-6 induced by sublethal H2O2 stress from human diploid fibroblasts. *Mol Cell Proteomics*. 2005;4:1273–1283. doi:10.1074/mcp.M500032-MCP200
44. Liu Y, Drozdov I, Shroff R, Beltran LE, Shanahan CM. Prelamin A accelerates vascular calcification via activation of the DNA damage response and senescence-associated secretory phenotype in vascular smooth muscle cells. *Circ Res*. 2013;112:e99–e109. doi:10.1161/CIRCRESAHA.111.300543
45. Acosta JC, O'Loghlen A, Banito A, et al.et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133:1006–1018. doi:10.1016/j.cell.2008.03.038
46. Abedin M, Lim J, Tang TB, Park D, Demer LL, Tintut Y. N-3 fatty acids inhibit vascular calcification via the p38-mitogen-activated protein kinase and peroxisome proliferator-activated receptor-gamma pathways. *Circ Res*. 2006;98:727–729. doi:10.1161/01.RES.0000216009.68958.e6
47. Couffinhal T, Duplàa C, Moreau C, Lamazière JM, Bonnet J. Regulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in human vascular smooth muscle cells. *Circ Res*. 1994;74:225–234. doi:10.1161/01.RES.74.2.225
48. Massberg S, Vogt F, Dickfeld T, Brand K, Page S, Gawaz M. Activated platelets trigger an inflammatory response and enhance migration of aortic smooth muscle cells. *Thromb Res*. 2003;110:187–194. doi:10.1016/S0049-3848(03)00342-6
49. Clément N, Gueguen M, Glorian M, et al.et al. Notch3 and IL-1beta exert opposing effects on a vascular smooth muscle cell inflammatory pathway in which NF-kappaB drives crosstalk. *J Cell Sci*. 2007;120(Pt 19):3352–3361. doi:10.1242/jcs.007872
50. Bennett BJ, Scatena M, Kirk EA, et al.et al. Osteoprotegerin inactivation accelerates advanced atherosclerotic lesion progression and calcification in older ApoE-/- mice. *Arterioscler Thromb Vasc Biol*. 2006;26:2117–2124. doi:10.1161/01.ATV.0000236428.91125.e6
51. Callegari A, Coons ML, Ricks JL, Rosenfeld ME, Scatena M. Increased calcification in osteoprotegerin-deficient smooth muscle cells: dependence on receptor activator of NF-κB ligand and interleukin 6. *J Vasc Res*. 2014;51:118–131. doi:10.1159/000358920
52. Shan Z, Yao C, Li ZL, et al.et al. Differentially expressed microRNAs at different stages of atherosclerosis in ApoE-deficient mice. *Chin Med J (Engl)*. 2013;126:515–520.
53. Raitoharju E, Lyytikäinen LP, Levula M, et al.et al. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. *Atherosclerosis*. 2011;219:211–217. doi:10.1016/j.atherosclerosis.2011.07.020
54. Fabbri M, Paone A, Calore F, et al.et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci USA*. 2012;109:E2110–E2116. doi:10.1073/pnas.1209414109
55. Fabbri M, Paone A, Calore F, Galli R, Croce CM. A new role for microRNAs, as ligands of Toll-like receptors. *RNA Biol*. 2013;10:169–174. doi:10.4161/rna.23144
56. He WA, Calore F, Londhe P, Canella A, Guttridge DC, Croce CM. Microvesicles containing miRNAs promote muscle cell death in cancer cachexia via TLR7. *Proc Natl Acad Sci USA*. 2014;111:4525–4529. doi:10.1073/pnas.1402714111