

# miR-34a Promotes Vascular Smooth Muscle Cell Calcification by Downregulating SIRT1 (Sirtuin 1) and Axl (AXL Receptor Tyrosine Kinase)

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**Objective**—Vascular calcification (VC) is age dependent and a risk factor for cardiovascular and all-cause mortality. VC involves the senescence-induced transdifferentiation of vascular smooth muscle cells (SMCs) toward an osteochondrogenic lineage resulting in arterial wall mineralization. miR-34a increases with age in aortas and induces vascular SMC senescence through the modulation of its target SIRT1 (sirtuin 1). In this study, we aimed to investigate whether miR-34a regulates VC.

**Approach and Results**—We found that miR-34a and Runx2 (Runt-related transcription factor 2) expression correlates in young and old mice. *Mir34a*<sup>+/+</sup> and *Mir34a*<sup>-/-</sup> mice were treated with vitamin D, and calcium quantification revealed that *Mir34a* deficiency reduces soft tissue and aorta medial calcification and the upregulation of the VC Sox9 (SRY [sex-determining region Y]-box 9) and Runx2 and the senescence p16 and p21 markers. In this model, miR-34a upregulation was transient and preceded aorta mineralization. *Mir34a*<sup>-/-</sup> SMCs were less prone to undergo senescence and under osteogenic conditions deposited less calcium compared with *Mir34a*<sup>+/+</sup> cells. Furthermore, unlike in *Mir34a*<sup>+/+</sup> SMC, the known VC inhibitors SIRT1 and Axl (AXL receptor tyrosine kinase) were only partially downregulated in calcifying *Mir34a*<sup>-/-</sup> SMC. Strikingly, constitutive miR-34a overexpression to senescence-like levels in human aortic SMCs increased calcium deposition and enhanced Axl and SIRT1 decrease during calcification. Notably, we also showed that miR-34a directly decreased Axl expression in human aortic SMC, and restoration of its levels partially rescued miR-34a-dependent growth arrest.

**Conclusions**—miR-34a promotes VC via vascular SMC mineralization by inhibiting cell proliferation and inducing senescence through direct Axl and SIRT1 downregulation, respectively. This miRNA could be a good therapeutic target for the treatment of VC.

**Visual Overview**—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2018;38:2079-2090. DOI: 10.1161/ATVBAHA.118.311298.)

**Key Words:** aging ■ humans ■ mice ■ senescence ■ vascular calcification

Vascular calcification (VC) is an age-related complication of atherosclerosis, type 2 diabetes mellitus, and chronic kidney disease and is characterized by the maladaptive transdifferentiation of vascular smooth muscle cells (VSMCs) toward an osteochondrogenic lineage, which results in hydroxyapatite deposition and eventually mineralization of the arterial wall.<sup>1,2</sup>

Although VC is a risk factor for cardiovascular and all-cause mortality, a therapy is not yet available, mostly because the pathways responsible for the VSMC osteochondrogenic phenotypic shift are still poorly understood.<sup>3</sup>

It has recently emerged that senescence and the acquisition of an inflammatory senescence-associated secretory phenotype increase the propensity of VSMC to experience the osteoblastic transition.<sup>4,5</sup> Remarkably, senescent VSMCs are characterized by the expression of bone-related genes, such as Runx2 (Runt-related transcription factor 2), alkaline phosphatase, and osteocalcin, and secretion of senescence-associated secretory phenotype molecules, such as IL-6 (interleukin 6), BMP2 (bone morphogenetic protein 2), and OPG (osteoprotegerin), that can induce the senescence and osteoblastic phenotype of neighboring VSMCs and local or circulating stem

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Nonstandard Abbreviations and Acronyms	
<b>Axl</b>	AXL receptor tyrosine kinase
<b>BMP2</b>	bone morphogenetic protein 2
<b>CDK</b>	cyclin-dependent kinase
<b>COMP</b>	cartilage oligomeric matrix protein
<b>HASMCs</b>	human aortic smooth muscle cells
<b>IL-6</b>	interleukin 6
<b>OPG</b>	osteoprotegerin
<b>Runx2</b>	Runt-related transcription factor 2
<b>SA-βgal</b>	senescence-associated β-galactosidase
<b>SIRT1</b>	sirtuin 1
<b>SMCs</b>	smooth muscle cells
<b>Sox9</b>	sex-determining region Y-box 9
<b>SRY</b>	sex-determining region Y
<b>VC</b>	vascular calcification
<b>VSMCs</b>	vascular smooth muscle cells
<b>vWF</b>	von Willebrand factor

cells.<sup>4–9</sup> Accordingly, during aging, the molecular mechanisms that favor VSMC senescence, like prelamin A accumulation<sup>4</sup> and ZMPSTE24<sup>10</sup> and COMP (cartilage oligomeric matrix protein)<sup>11,12</sup> downregulation, promote VSMC osteogenic differentiation as well.

miRNAs are negative post-transcriptional regulators of gene expression and have potential as therapeutic targets.<sup>13</sup> Although they have been implicated in several cardiovascular biological processes, the role of miRNAs in VC is still largely unexplored.<sup>14</sup> miR-34a was firstly described as a tumor suppressor that can regulate cancer cell proliferation, apoptosis, and senescence.<sup>15</sup> Notably, miR-34a plays an important role during the aging process.<sup>16,17</sup> Indeed, its expression increases in different aged organs and tissues,<sup>6,16,18–20</sup> and its genetic ablation or inhibition in mice has been shown to improve cardiac performance and ventricular remodeling in the aged heart.<sup>16</sup> Notably, miR-34a is a senescence-inducer miRNA<sup>21</sup> and can mediate endothelial and endothelial progenitor cell senescence through the modulation of its target—the longevity-associated gene SIRT1 (sirtuin 1).<sup>18,22</sup> We have recently reported that miR-34a is upregulated in aged mouse aortas, promoting VSMC senescence through the direct downregulation of SIRT1 and the expression of a subset of senescence-associated secretory phenotype factors, including the pro-osteogenic molecules BMP2 and IL-6.<sup>6</sup> Interestingly, Takemura et al<sup>5</sup> have demonstrated that SIRT1 acts as a VC inhibitor by counteracting VSMC senescence and their consequent calcification. Notably, miR-34a can regulate other VSMC calcification inhibitors, for instance, the antiapoptotic receptor tyrosine kinase Axl (AXL receptor tyrosine kinase) has been described as a miR-34a target in solid cancer and able to inhibit VSMC calcium deposition *in vitro*.<sup>23,24</sup>

Given the association among miR-34a, senescence, and calcification, we hypothesized that the age-dependent upregulation of this miRNA may drive the senescence-induced VSMC osteochondroblastic changes and thereby VC. We found that miR-34a deficiency *in vivo* reduces soft tissue, including aorta, calcification by preventing the expression of

the VC markers Sox9 (SRY [sex-determining region Y]-box 9) and Runx2, as well as of the senescence factors p16 and p21. Expression of miR-34a increases before aorta mineralization. Smooth muscle cell (SMC) isolated from *Mir34a*<sup>-/-</sup> mice shows reduced senescence and calcification and slightly higher expression of SIRT1 and Axl during the mineralization process induced by hyperphosphatemia. Strikingly, miR-34a-overexpressing human aortic SMCs (HASMCs) exhibit increased senescence and calcium deposition along with lower levels of SIRT1 and Axl. We also demonstrated that miR-34a directly targets Axl in HASMC and inhibits their proliferation, at least in part, through Axl downregulation.

This study suggests that miR-34a is a promoter of VC, by inducing VSMC growth arrest and senescence via direct Axl and SIRT1 downregulation, respectively. In the future, a therapeutic strategy targeted against miR-34a might be considered for the treatment or prevention of age-associated VC.

## Materials and Methods

The authors declare that all supporting data are available within the article and its [online-only Data Supplement](#).

### Animal Experiments

Animal work was performed in conformity with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and in accordance with experimental protocols approved by the University Committee on Animal Resources at the University of Milan (734–2015). Mice were housed in standard cages on a 12:12-hour light-dark cycle and fed a normal chow diet *ad libitum*. JAX C57BL/6J mice (*Mir34a*<sup>+/+</sup>, wild type) were purchased from Charles River Laboratories International, Inc. (stock No. 000664; Wilmington, MA). *Mir34a*<sup>-/-</sup> mouse line was already generated<sup>25</sup> and purchased from The Jackson Laboratory (stock No. 018279; Bar Harbor, ME).

Only male mice were included in this study because there are evidences from the literature showing sex differences as the estrogen hormone protects females from VC.<sup>26–28</sup> Twelve-week-old male *Mir34a*<sup>-/-</sup> and *Mir34a*<sup>+/+</sup> were treated with either 500 000 IU·kg<sup>-1</sup>·d<sup>-1</sup> vitamin D (cholecalciferol, C1357; Sigma-Aldrich, St. Louis, MO) or a mock solution (1% [v/v] ethanol, 7% [v/v] Kolliphor EL, and 3.75% [wt/vol] dextrose, all from Sigma-Aldrich) administered subcutaneously for 3 consecutive days and euthanized 7 days after the first injection.<sup>29,30</sup> Animals were anesthetized with an intraperitoneal injection of ketamine:medetomidine cocktail (100:10 mg/kg) and perfused with PBS from the apex of the heart. Blood was collected by cardiac puncture; aortas, hearts, lungs, and kidneys were dissected out and processed for aortic medial thickness and cellular density measurement, calcium content quantification, von Kossa staining, and immunohistochemistry as described in the [online-only Data Supplement](#). A semiquantitative calcification score was also determined.<sup>31</sup> Calcification on arterial cross section at 3 different levels for every mouse was scored using the following system: 0, no calcification; 1, focal calcification spots; 2, partial calcification covering 20% to 80% of the arterial circumference; and 3, circumferential calcification.

For the aging experiments, aortas were isolated from C57BL/6J male young (2.5 months old) and old (21 months old) mice and immediately frozen for RNA and protein extraction or paraffin embedded for Alizarin Red staining as described in the [online-only Data Supplement](#).

### Cell Culture

HASMCs were purchased from Lonza (Basel, Switzerland) and cultured in SmGM-2 complete medium (Lonza). The cell donors were white men of 22, 30, and 43 years of age.

Murine SMCs were isolated as already described.<sup>32</sup> Immunofluorescence analyses confirmed that isolated cells express  $\alpha$ -smooth muscle actin while were not positive for the endothelial marker vWF (von Willebrand factor; Figure IIIA and IIIB in the [online-only Data Supplement](#)).

Cells were transfected and infected as described in the [online-only Data Supplement](#).

### Calcification Assay

Cells were cultured in osteogenic medium (DMEM supplemented with 15% fetal bovine serum, 5 mmol/L phosphate, 10 mmol/L sodium pyruvate, and 50  $\mu$ g/mL ascorbic acid) for 3 or 7 days. To quantify the precipitated calcium, cells grown in 12-well plates were incubated overnight with 250  $\mu$ L of 0.6 N HCl at 4°C, and then, supernatants were collected. To extract protein for normalization, cells were incubated overnight at 4°C with 250  $\mu$ L of 0.1% SDS-0.1 N NaOH lysis buffer. The precipitated calcium was quantified by colorimetric analysis with the QuantiChrom Calcium Assay Kit (DICA-500; Gentaur, Kampenhout, Belgium), whereas the protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Cells undergone calcification were also processed for quantitative reverse transcriptase polymerase chain reaction and Western blot analyses as described in the [online-only Data Supplement](#).

### Statistical Analysis

In vitro experiments were performed at least 3 $\times$ . Data were analyzed with GraphPad Prism software, version 7 (GraphPad Software, Inc, La Jolla, CA). The Shapiro-Wilk test was used to assess the normality of distribution of investigated parameters. Differences between 2 groups were analyzed with unpaired Student *t* test or Mann-Whitney *U* test for normally or not normally distributed variables, respectively, otherwise stated in the figure legends. Statistical analysis between >2 groups was conducted by 1- or 2-way ANOVA with Bonferroni post hoc test, as reported in the figure legends. A value of *P* < 0.05 was considered statistically significant; values are presented as mean $\pm$ SE.

## Results

### Mir34a Genetic Ablation Reduces Soft Tissue and VC In Vivo

We have previously demonstrated that miR-34a levels increase in the aortas of aged mice along with senescence-associated proteins, such as p16 and p21, and that miR-34a induces VSMC senescence.<sup>6</sup> Old aortas also show signs of calcification when compared with young ones (Figure IA and IB in the [online-only Data Supplement](#)).<sup>33,34</sup> Hence, as a prerequisite of our study, we correlated the expression of miR-34a and the VC marker Runx2 in aortas of young (2.5 months old) and old (21 months old) mice. Both miR-34a and Runx2 were upregulated in aortas of aged animals, with a positive association (Figure IC through IE in the [online-only Data Supplement](#)). Accordingly, Runx2 protein levels increased in old aortas (Figure IF in the [online-only Data Supplement](#)).

To evaluate whether miR-34a could play a role in VC, *Mir34a*<sup>+/+</sup> and *Mir34a*<sup>-/-</sup> mice were subcutaneously injected for 3 consecutive days with a toxic dose of vitamin D or a mock solution (control [Ctrl]) and euthanized 7 days after the first injection.<sup>29,30</sup> Although *Mir34a*<sup>+/+</sup> and *Mir34a*<sup>-/-</sup> animals showed a slightly different body weight before the treatment (Figure IIA in the [online-only Data Supplement](#)), weight loss on vitamin D treatment was significantly lower in *Mir34a*<sup>-/-</sup> than in *Mir34a*<sup>+/+</sup> mice, indicating a major discomfort in the latter group (Figure IA). As expected, calcium levels increased

in the sera of vitamin D-treated mice when compared with the corresponding control group; interestingly, serum calcium in vitamin D-treated *Mir34a*<sup>-/-</sup> animals was lower than those in corresponding *Mir34a*<sup>+/+</sup> mice (Figure 1B). We quantified calcium deposition in soft tissues, including kidneys, lungs, hearts, and aortas. Whereas calcium deposition was negligible in control mice, it significantly raised in vitamin D-treated mice; notably, the genetic ablation of *Mir34a* reduced calcification in all tested organs (Figure 1C).

To investigate VC, we further characterized aortas of these animals. Aortas of *Mir34a*<sup>-/-</sup> mice showed a basal higher medial thickness and cellular density than *Mir34a*<sup>+/+</sup> (Figure IIB through IID in the [online-only Data Supplement](#)). Then, we assessed calcium deposition by von Kossa staining on aortic sections. In comparison with *Mir34a*<sup>+/+</sup> animals, fewer *Mir34a*<sup>-/-</sup> mice accumulated calcium in the medial arterial layer and to a lesser extent after vitamin D administration (Figure 2A through 2C). We also analyzed by immunohistochemistry the expression pattern of 2 well-known VC makers, Runx2 and Sox9,<sup>2,31</sup> as well as of p16 and p21; we observed that only *Mir34a*<sup>+/+</sup> mice displayed positive staining in VSMC nearby the calcified regions (Figure 2D).

Altogether, our data demonstrate that *Mir34a* deficiency reduces soft tissue and VC induced by an overdose of vitamin D in vivo. In the aorta, *Mir34a* deficiency prevented senescence and transdifferentiation of VSMC.

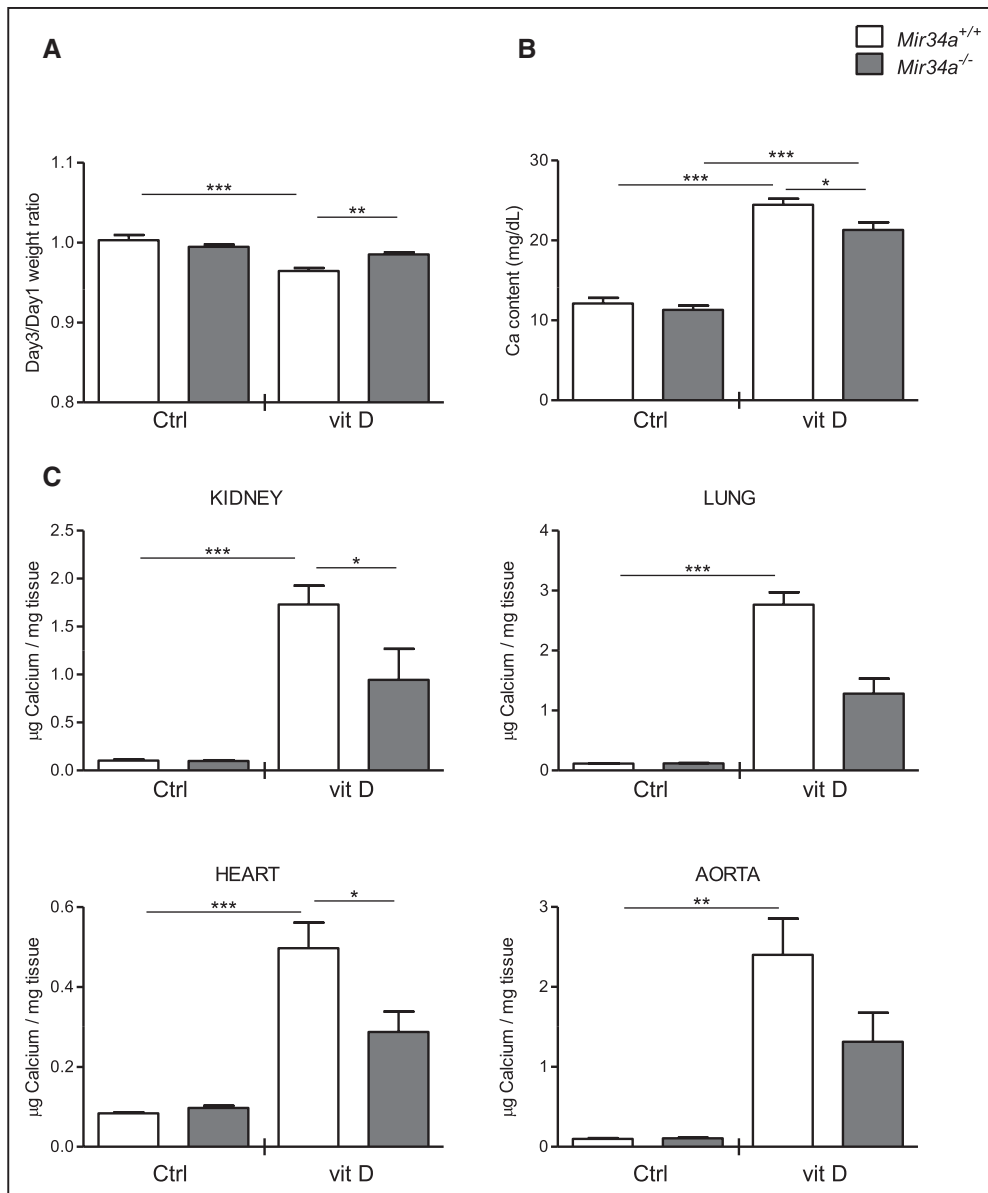
### miR-34a Is Upregulated in Aortas That Display Features of Senescence Before Overt Calcification

To gain further insight in the role of miR-34a in VC, we determined its expression in aortas and serum of wild-type mice during the mineralization process induced by vitamin D. In the aorta, calcium deposition was detectable at day 5 and was significantly higher at day 7 after the first injection of vitamin D in comparison with the corresponding mice treated with mock solution (Ctrl; Figure 3A). Notably, an induction of miR-34a by vitamin D was already evident at day 3 but soon decreased to the levels of Ctrl at day 5 (Figure 3B). Interestingly, p21 mRNA was markedly upregulated at days 3 and 5 (Figure 3C). No significant differences in circulating miR-34a levels were detected between vitamin D-treated and Ctrl mice at any time point, although a trend to a decrease in its amount was observed during the progression of calcification (Figure 3D).

These results indicate that in vivo vitamin D treatment induces a transient upregulation of miR-34a that is concomitant with p21 induction and precedes overt aortic calcification.

### Mir34a Genetic Ablation Decreases SMC Senescence and Calcium Deposition Ex Vivo

To better investigate the possible role of miR-34a in the SMC osteochondrogenic transition, we isolated SMC from *Mir34a*<sup>+/+</sup> and *Mir34a*<sup>-/-</sup> mice (Figure IIIA and IIIB in the [online-only Data Supplement](#)). In accordance with our previously published data<sup>6</sup> and aorta cell density data (Figure IIB and IID in the [online-only Data Supplement](#)), *Mir34a*<sup>-/-</sup> cells displayed a higher proliferation rate than *Mir34a*<sup>+/+</sup> cells (Figure IIIC in the [online-only Data Supplement](#));



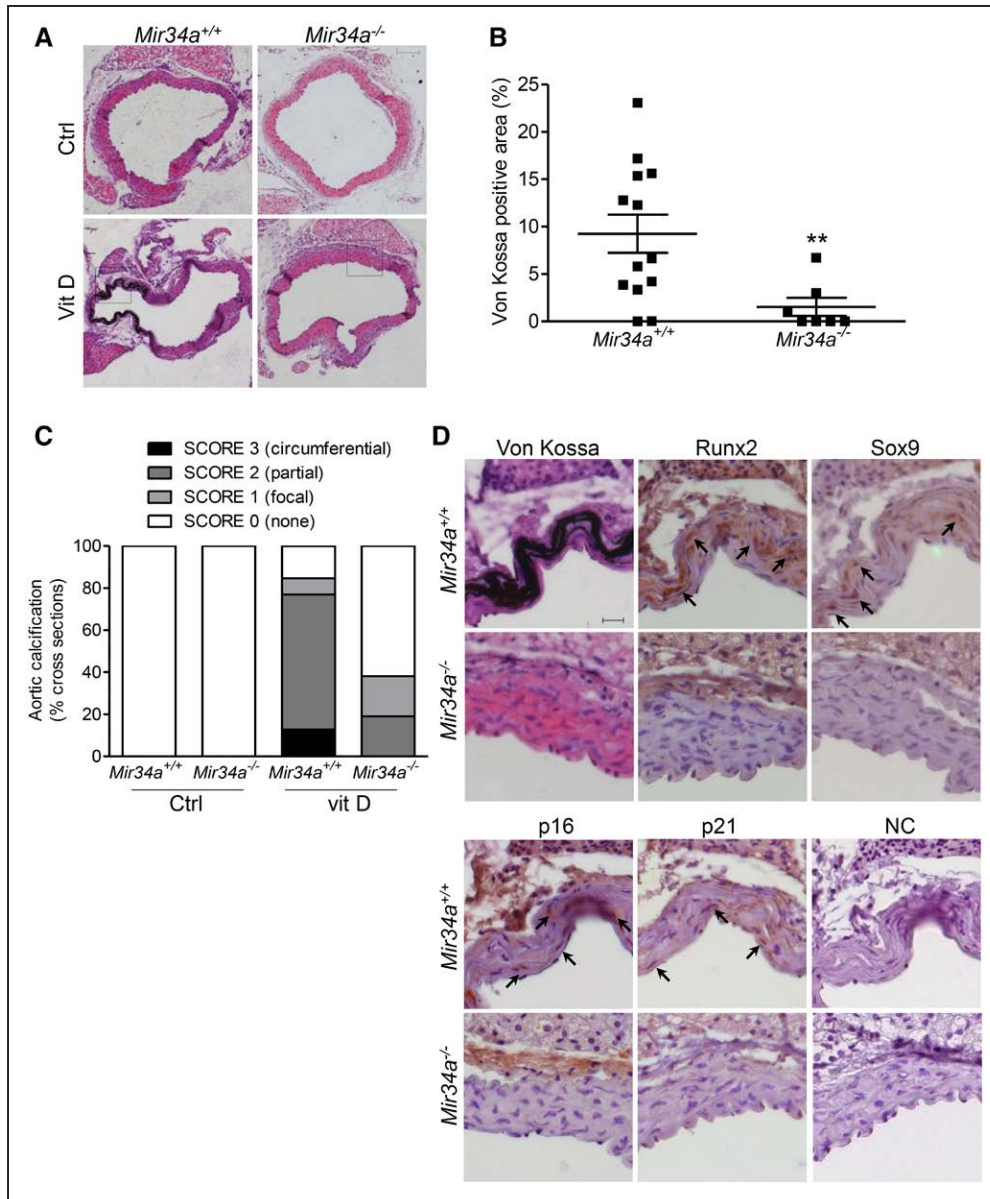
**Figure 1.** *Mir34a* genetic ablation reduces soft tissue calcification. **A–C**, Twelve-week-old *Mir34a*<sup>-/-</sup> and *Mir34a*<sup>+/+</sup> mice were treated subcutaneously with either vitamin D (vit D) or a mock solution (control [Ctrl]) for 3 consecutive days and euthanized 7 d (day 7) after the first injection. **A**, Mice weight ratio at the third day (day 3) and before the first vit D injection (day 1; n=6, 5, 13, and 8). **B**, Calcium content in the sera at day 7 was quantified by a colorimetric analysis (n=9, 8, 17, and 11). **C**, Calcium content in the indicated organs and tissue was quantified by the colorimetric analysis (n=6, 5, 12–13, and 8). Values are mean±SE. One-way ANOVA with Bonferroni post hoc test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.0001.

furthermore, they showed lower SA-βgal (senescence-associated β-galactosidase) activity and p16 expression, when compared with *Mir34a*<sup>+/+</sup> cells (Figure IIID and IIIE in the [online-only Data Supplement](#)). We induced *Mir34a*<sup>+/+</sup> and *Mir34a*<sup>-/-</sup> SMC calcification by culturing them for 7 days in an osteogenic medium containing a pathological concentration of inorganic phosphate. In accordance with our in vivo results, *Mir34a*<sup>-/-</sup> SMCs deposited lower levels of calcium relative to *Mir34a*<sup>+/+</sup> cells (Figure 4A).

To get deeper insight in the molecular pathways affected by miR-34a that promote SMC calcification, we evaluated the expression of the VC inhibitors Axl and SIRT1.<sup>5,23</sup> We have previously demonstrated that miR-34a directly targets SIRT1 and promotes senescence in HASMC,<sup>6</sup> whereas Axl is known

to be targeted by miR-34a in other cell types.<sup>24</sup> Interestingly, during SMC mineralization, Axl mRNA level was higher in *Mir34a*<sup>-/-</sup> SMCs in respect to *Mir34a*<sup>+/+</sup> cells at day 3 (Figure 4B), whereas SIRT1 mRNA expression was not significantly increased (Figure 4C). At day 7, both transcripts were not influenced by the absence of *Mir34a* (Figure 4B and 4C). Both SIRT1 and Axl proteins were downregulated in *Mir34a*<sup>+/+</sup> cells at day 7 when compared with day 3, whereas they were not or less modulated in *Mir34a*<sup>-/-</sup> SMCs, respectively (Figure 4D through 4F).

These results demonstrate that SMCs lacking miR-34a expression have higher proliferation rate, reduced propensity to undergo senescence and deposit calcium, and show higher expression of the VC inhibitors SIRT1 and Axl.

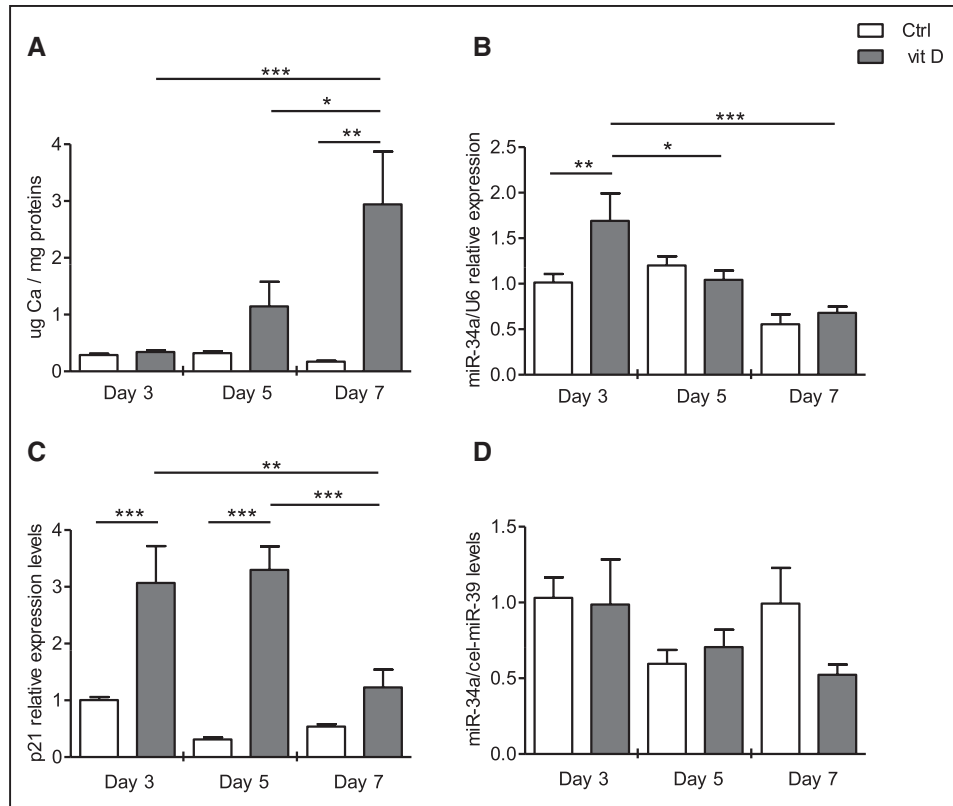


**Figure 2.** *Mir34a* genetic ablation reduces vascular calcification. **A**, Representative images of von Kossa staining (black) on sections from distal thoracic aortas. Nuclei were counterstained with hematoxylin (purple); scale bar=100 μm. **B**, Bars show the percentage of von Kossa-positive area to the total aortic area of vitamin D (Vit D)-treated mice (n=13 and 7). Values are mean±SE. Mann-Whitney U test. \*\*P<0.01. **C**, Evaluation of vascular calcification by a semiquantitative scoring of von Kossa-stained aortic sections of vit D-treated and (control [Ctrl]) mice. **D**, Enlarged images of the von Kossa-stained sections shown in A and adjacent sections stained with antibodies specific for the indicated proteins or nothing (negative control [NC]) of vit D-treated mice (brown, black arrows; scale bar=20 μm). Nuclei were counterstained with hematoxylin (purple). Runx2 indicates Runt-related transcription factor 2; and SA-βgal, senescence-associated β-galactosidase.

**miR-34a Inhibits VSMC Proliferation by Directly Targeting Axl in HASMC**

Next, we verified whether miR-34a can specifically modulate Axl in VSMC. We transfected HASMC with a miR-34a mimic or a mimic negative control; miR-34a overexpression downregulates Axl protein levels already at 24 hours post-transfection (Figure 5A). Accordingly, Axl expression was higher in HASMC transfected with a miR-34a inhibitor compared with the scramble (SCR) control (Figure 5B). To prove that miR-34a directly affects Axl expression, we transfected HASMCs with a vector carrying Axl cDNA devoid of the 3'-UTR (untranslated region) containing miR-34a seed

sequence<sup>24</sup> or an empty vector, together with a miR-34a mimic or a mimic negative control. Western blot analysis confirmed that the endogenous Axl was severely lowered upon miR-34a overexpression, whereas the exogenous Axl was unaffected by miR-34a ectopic expression (Figure 5C). Because Axl regulates cell proliferation and survival,<sup>35</sup> we determined whether its downregulation mediated by miR-34a could affect HASMC growth. As expected, miR-34a reduced cell number 72 hours after transfection (empty miR-34a versus empty SCR; Figure 5D); Axl ectopic expression alone increased the number of HASMC (Axl SCR versus empty SCR; Figure 5D) and, in combination with miR-34a, partially



**Figure 3.** miR-34a is upregulated in aortas of vitamin D (vit D)-treated mice that display features of senescence before overt calcification. Twelve-week-old wild-type mice were treated subcutaneously with either vit D or a mock solution (control [Ctrl]) for 3 consecutive days and euthanized 3 (day 3), 5 (day 5), or 7 (day 7) d after the first injection. **A**, Calcium content in the aortas was quantified by colorimetric analysis (n=5, 5, 5, 3, and 5). **B** and **C**, miR-34a and p21 expression in the aortas was analyzed by quantitative reverse transcriptase polymerase chain reaction and normalized to corresponding U6 and Hprt levels, respectively (n=5, 5-3, 5, 5-4, 3, and 5). **D**, miR-34a levels in the sera of mice were determined at the indicated days (n=5, 4, 5, 3, and 5). Values are mean±SE. Two-way ANOVA with Bonferroni post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ .

reversed miR-34a-mediated inhibition of cell proliferation (Axl miR-34a versus empty miR-34a; Figure 5D).

All together, these data indicate that miR-34a inhibits VSMC proliferation to some extent through the direct modulation of Axl.

### miR-34a-Induced Senescence Enhances HASMC Calcification Through Axl and SIRT1 Downregulation

We previously demonstrated that proliferative/young HASMCs express lower miR-34a levels compared with old/senescent HASMC, and transient miR-34a overexpression in young cells induces senescence.<sup>6</sup> Because old/senescent HASMC calcify more compared with young cells,<sup>5,36</sup> we verified whether miR-34a-induced VSMC senescence enhances VC. First, we set up an in vitro model of proliferative/young HASMC calcification induced by hyperphosphatemia. A significant augmentation of calcium deposition was observed by von Kossa staining and colorimetric analysis (Figure 4A and 4B in the [online-only Data Supplement](#)) along with an upregulation of VC markers alkaline phosphatase and Runx2 during the calcification process (Figure 4C through 4E in the [online-only Data Supplement](#)).

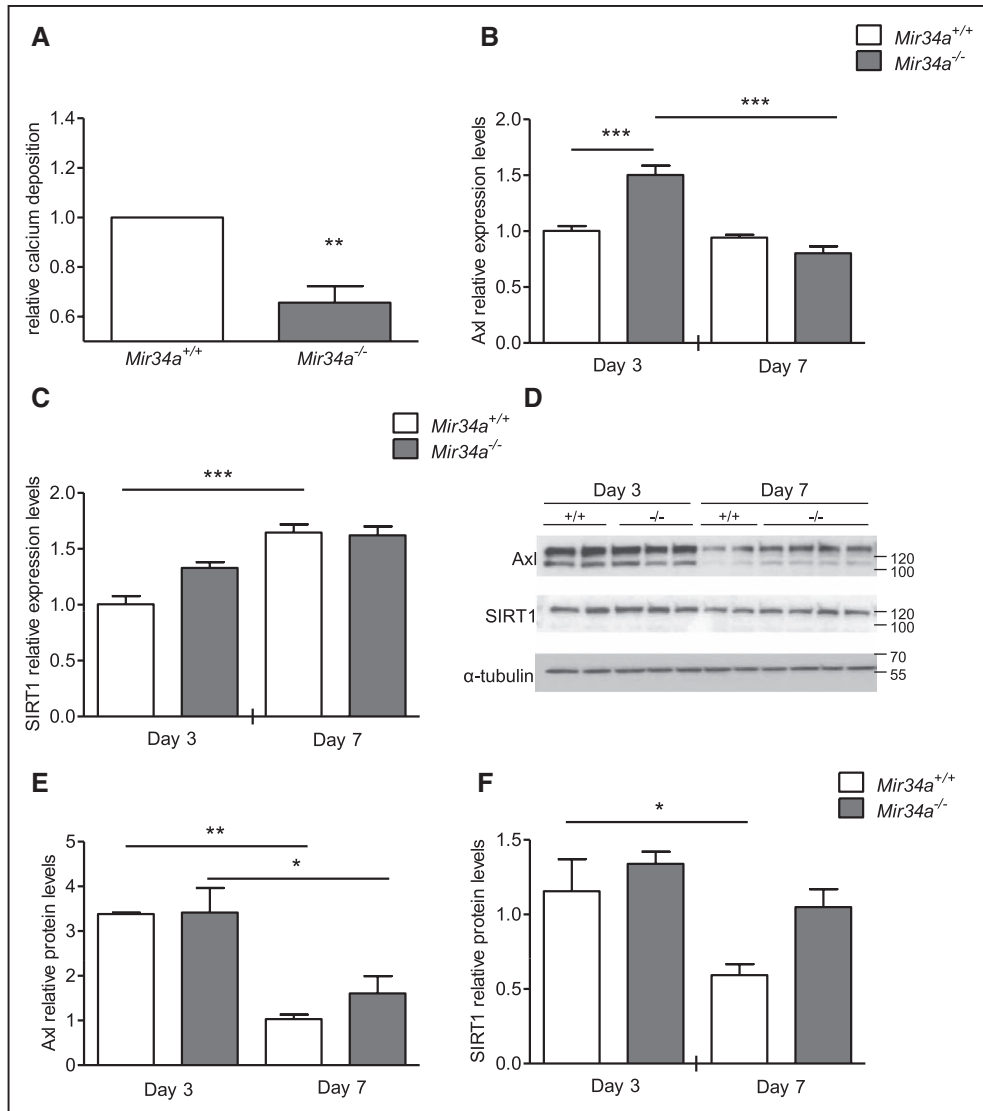
Then, we confirmed in our experimental conditions that senescent HASMC (passage 15) expressing higher levels of miR-34a mineralize more than young cells (passage

5; Figure 6A). Thus, we used a lentiviral infection to stably express miR-34a in proliferative HASMC to a level comparable with senescent HASMC (Figure 6A in the [online-only Data Supplement](#)). Indeed, miR-34a-overexpressing HASMCs had greater SA- $\beta$ gal activity, increased or decreased levels of the growth arrest marker p21 or Axl and SIRT1 mRNA, respectively (Figure 6B through 6E in the [online-only Data Supplement](#)). Strikingly, miR-34a-overexpressing HASMCs evidenced a rise in calcium deposition at day 7 of calcification, which was not seen in scramble control cells (SCR; Figure 6B).

Hence, we assessed Axl and SIRT1 expression during the mineralization process. Axl mRNA levels were lower in miR-34a-overexpressing HASMC at both days 3 and 7 in respect to SCR cells, whereas no major differences were detected for the SIRT1 transcript (Figure 6C and 6D). Western blot analysis revealed that both proteins decreased significantly at day 3 of calcification in miR-34a-overexpressing cells compared with control SCR HASMC (Figure 6E through 6G).

Finally, we determined miR-34a expression in HASMC at different days of culture in the osteogenic medium observing no significant modulation of its levels during the osteochondrogenic transition (Figure 6I in the [online-only Data Supplement](#)).

Taken together, our findings show that miR-34a upregulation is necessary to induce HASMC growth arrest and



**Figure 4.** *Mir34a* genetic ablation significantly decreases smooth muscle cell (SMC) senescence and calcium deposition in vitro. **A–F**, SMCs were isolated from *Mir34a*<sup>-/-</sup> and *Mir34a*<sup>+/+</sup> mice. **A**, Cells were cultured in the osteogenic medium for 7 d, and the amount of precipitated calcium was quantified by colorimetric analysis (n=7). Relative calcium levels of *Mir34a*<sup>-/-</sup> to corresponding *Mir34a*<sup>+/+</sup> SMC were shown. Values are mean±SE. \*\**P*<0.01. Paired *t* test. **B–F**, Cells were cultured in the osteogenic medium for 3 or 7 d (days 3 and 7). **B** and **C**, Axl (AXL receptor tyrosine kinase) and SIRT1 (sirtuin 1) mRNA levels were analyzed by quantitative reverse transcriptase polymerase chain reaction and normalized to corresponding *Hprt* levels (n=3, 3, 4, and 3). **D–F**, Protein extracts were subjected to Western blot analysis with anti-Axl, anti-SIRT1, or anti- $\alpha$ -tubulin (loading control) antibodies. **E** and **F**, Bars show quantification of normalized densitometric ratios (n=3, 4, 4, and 5). Values are mean±SE. One-way ANOVA with Bonferroni post hoc test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.0001.

senescence to promote calcification through downregulation of its targets Axl and SIRT1.

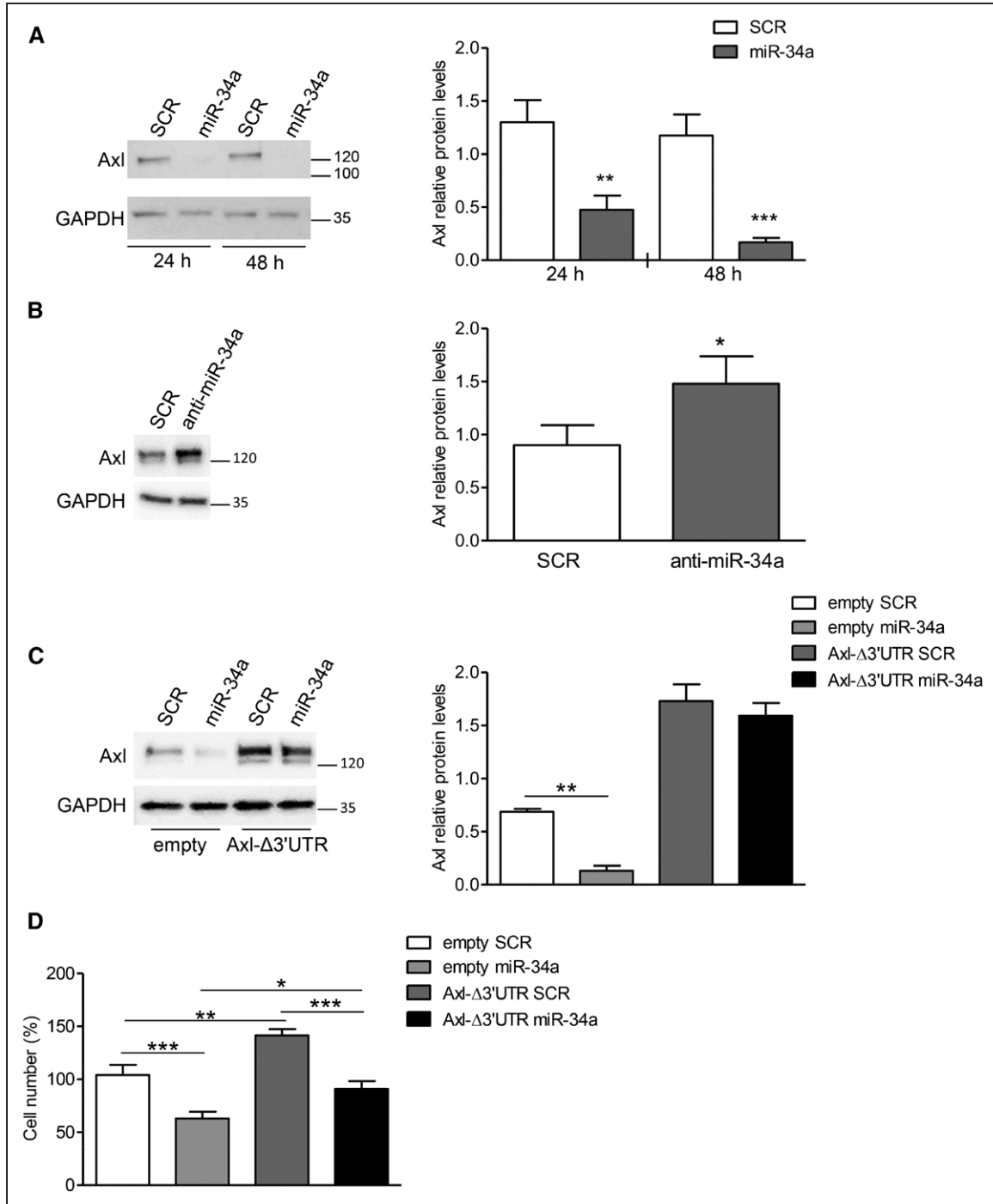
### Discussion

VC is a pathology that occurs frequently in the elderly population and is associated with atherosclerosis, type 2 diabetes mellitus, and chronic kidney disease, which are diseases displaying features of premature aging.<sup>8,37,38</sup> Although the subjects with VC are high-risk patients, an effective therapy is not yet available because of the poor understanding of the molecular mechanisms underlying this complication.<sup>3</sup>

We recently published that miR-34a is upregulated in aged murine aortas and that an increase of its levels in VSMC induces growth arrest, senescence, and the expression of certain senescence-associated secretory phenotype factors,

including the pro-osteogenic molecules BMP2 and IL-6.<sup>6</sup> In the present study, we demonstrated for the first time that the aging-associated miR-34a is a VC promoter.

We confirmed previous data reporting that aortas of aged mice show signs of calcification along with increased expression of VC markers, such as Runx2 (Figure 1A, 1B, 1D, and 1F in the [online-only Data Supplement](#)).<sup>33,34</sup> Using an established mouse model of soft tissue and aortic medial layer calcification induced by an overdose of vitamin D,<sup>29,30</sup> we demonstrated that *Mir34a* gene deletion prevents VC in vivo. miR-34a deficiency significantly reduced calcium levels in the serum and all tested organs and, with particular regard to aortas, prevented the induction of Runx2 and Sox9 and aortic VSMC osteochondrogenic transdifferentiation (Figures 1 and 2). Expression of p16 and p21 was found only around the calcified regions of

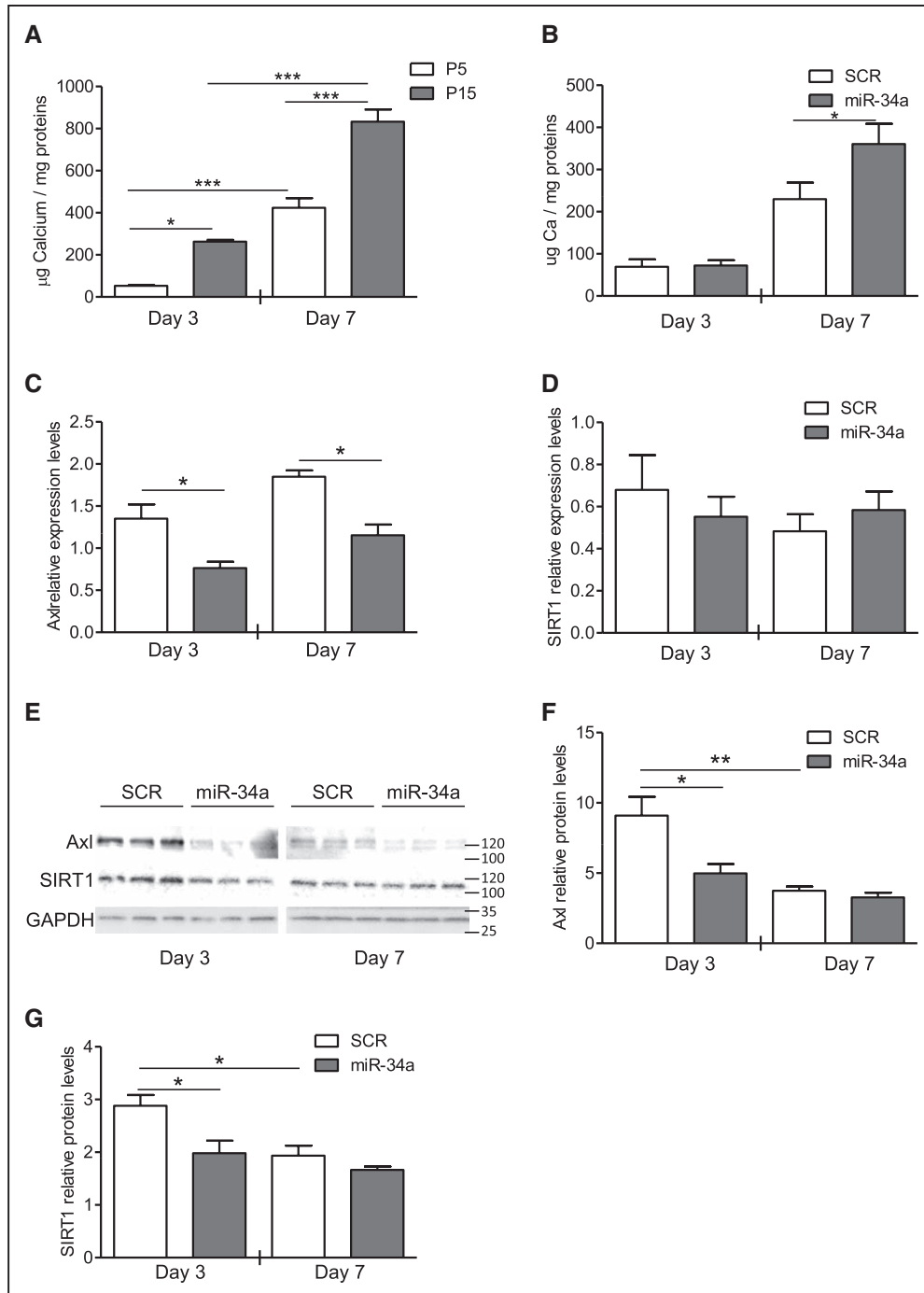


**Figure 5.** Axl (AXL receptor tyrosine kinase) expression is directly modulated by miR-34a in human aortic smooth muscle cells (HASMC). **A**, HASMC were transfected with a miR-34a mimic (miR-34a) or a mimic control (scramble, SCR) and cultured for 24 or 48 h. Protein extracts were analyzed with anti-Axl or anti-GAPDH (loading control) antibody (left). Bars show quantification of normalized densitometric ratios (n=4 and 5; right). **B**, HASMCs were transfected with a miR-34a hairpin inhibitor (anti-miR-34a) or a hairpin inhibitor negative control (SCR) and cultured for 72 h. Protein extracts were analyzed with anti-Axl or anti-GAPDH (loading control) antibody (left). Bars show normalized densitometric ratios (n=4; right). **C** and **D**, HASMCs were transfected with miR-34a or SCR along with either a 3' untranslated region-deleted Axl-expression vector (Axl-Δ3'UTR) or an empty vector (empty). **C**, Twenty-four hours after transfection, protein extracts were analyzed with anti-Axl or anti-GAPDH (loading control) antibody (left). Bars show quantification of normalized densitometric ratios (n=3; right). **D**, The graph shows the cell number at 72 h after transfection (n=5). Values are mean±SE. Student *t* test or 1-way ANOVA with Bonferroni post hoc test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.0001.

aortas of *Mir34a*<sup>+/+</sup> mice (Figure 2D) that is in line with previously published data showing that these 2 senescence markers are upregulated in calcified rodent aortas, human arteries, and VSMCs.<sup>4,5,39–42</sup> Notably, after vitamin D treatment, miR-34a

levels raised in a transient manner before a detectable amount of calcium was deposited in the aorta and along with p21 induction (Figure 3A through 3C) suggesting that in vivo miR-34a upregulation is necessary to promote tissue senescence





**Figure 6.** miR-34a overexpression increases human aortic smooth muscle cell (HASMC) calcium deposition. **A**, Calcium deposition quantification with the colorimetric analysis at 3 and 7 d of culturing in osteogenic medium of HASMC at a proliferative passage (P5) and at senescence (passage 15 [P15]). **B–G**, HASMCs were infected with either pMIRNA1 (SCR) or pMIRH34a (miR-34a) lentivirus at an MOI (multiplicity of infection) of 10 and cultured in growth medium for 48 h. Then, they were cultured in the osteogenic medium for 3 (day 3) or 7 (day 7) d. **B**, The amount of precipitated calcium was quantified by colorimetric analysis (n=7). **C** and **D**, Axl (AXL receptor tyrosine kinase) and SIRT1 (sirtuin 1) mRNA levels were analyzed by quantitative reverse transcriptase polymerase chain reaction and normalized to corresponding GAPDH levels (n=3). **E**, Protein extracts were analyzed with anti-Axl, anti-SIRT1, or anti-GAPDH (loading control) antibody. **F** and **G**, Bars show quantification of normalized densitometric ratios (n=3). Values are mean±SE. One-way ANOVA with Bonferroni post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ .

that eventually triggers the onset and progression of calcification. In accordance, it has been shown that the activation of the senescence program by a calcification stimulus precedes and controls VSMC mineralization process,<sup>43</sup> and senescence still occurs when osteogenesis is blocked by silencing specific calcification factors like Runx2.<sup>10</sup>

Our in vitro data support the in vivo findings. Senescent VSMCs that express higher amount of miR-34a are more prone to calcify in response to high phosphate compared with younger cells (Figure 6A)<sup>5,6,36</sup> and miR-34a-induced senescence facilitates calcification mediated by hyperphosphatemia. Indeed, murine *Mir34a*<sup>-/-</sup> SMCs displayed decreased

senescence features, as revealed by lower SA- $\beta$ gal staining signal and p16 expression, and reduced calcium deposition when compared with *Mir34a*<sup>+/+</sup> cells (Figure IIID and IIIE in the [online-only Data Supplement](#); Figure 4A); conversely, constitutive overexpression of miR-34a to senescence-like levels in HASMC enhanced calcification (Figure VA through VC in the [online-only Data Supplement](#); Figure 6B). Furthermore, miR-34a is not modulated during HASMC mineralization (Figure VI in the [online-only Data Supplement](#)). Hence, miR-34a being an inducer of VSMC growth arrest and senescence is involved in the early mechanisms indispensable for VSMC osteochondrogenic transdifferentiation.

Interestingly, our in vitro experiments showed milder effects of miR-34a on SMC calcification compared with the in vivo results. The difference may depend on the contribution of other cell types to VC. In fact, it has been shown that pericytes, endothelial cells, fibroblasts, and resident and circulating progenitor cells can differentiate toward an osteochondrogenic lineage as well and thereby participate to the calcification process.<sup>44</sup> Hence, the low propensity to VC of *Mir34a*<sup>-/-</sup> mice could be explained by the prevention of the osteochondrogenic transition of other cell types in addition to SMC.

Mechanistically, we identified 2 VC inhibitors, SIRT1 and Axl,<sup>5,23</sup> as miR-34a targets during the calcification process. It has been already shown that SMC osteoblastic transdifferentiation is characterized by SIRT1 downregulation, and maintaining of high levels of this protein prevents SMC calcification through senescence inhibition.<sup>5</sup> We have already proven that SIRT1 is a direct miR-34a target in HASMC and that its miR-34a-mediated downregulation enhances senescence in these cells.<sup>6</sup> In this study, we further show that the direct modulation of SIRT1 by miR-34a influences calcification of VSMC. In fact, unlike wild-type murine SMCs, SIRT1 protein levels were not significantly altered in *Mir34a*<sup>-/-</sup> cells under high phosphate conditions (Figure 4D and 4F), whereas they were significantly reduced at an early stage of calcification in miR-34a-overexpressing HASMCs (Figure 6E and 6G). A similar behavior was found for Axl (Figures 4D, 4E, 6E, and 6F).

Cells must arrest their cell cycle before undergoing senescence.<sup>40</sup> miR-34a inhibits HASMC proliferation by blocking the G1- to S-phase transition along with the upregulation of p21 protein levels before SIRT1 downregulation.<sup>6</sup> Herein, we demonstrated that *Mir34a*<sup>-/-</sup> aortas display a higher VSMC density and isolated *Mir34a*<sup>-/-</sup> SMC, an enhanced proliferative rate (Figures IIB through IID and IIIC in the [online-only Data Supplement](#)) further corroborating miR-34a as an important regulator of VSMC proliferation. Furthermore, we validated Axl as a miR-34a target in HASMC and found that miR-34a downregulates Axl with a faster kinetic compared with SIRT1 (24 versus 48 hours) and alongside with p21 upregulation and cell cycle arrest (Figure 5A through 5C).<sup>6</sup> Axl signaling has been shown to control several VSMC functions including proliferation,<sup>45</sup> migration,<sup>46</sup> and cell survival<sup>47</sup>; furthermore, Axl is also known to be downregulated during cell mineralization and its overexpression inhibits VSMC calcification via activation of the antiapoptotic PI3K (phosphatidylinositol 4,5-bisphosphate 3-kinase)/AKT (RAC- $\alpha$  serine/threonine-protein kinase) pathway.<sup>23</sup> Our findings demonstrate that miR-34a, at least in part, inhibits HASMC proliferation by directly reducing Axl

protein levels (Figure 5C and 5D) and that under a procalcification stimulus, miR-34a-dependent Axl decrease favors SMC osteochondrogenic transition (Figures 4D, 4E, 6E, and 6F). The mild effect of Axl modulation on HASMC proliferation is likely because miR-34a is known to influence cell growth by targeting also several regulators of the cell cycle machinery, such as CDKs (cyclin-dependent kinases) and cyclins.<sup>48-51</sup>

At the molecular level, miRNAs bind mRNA targets to mediate RNA degradation or inhibit protein translation.<sup>52</sup> We found that both Axl and Sirt1 mRNAs diminish in HASMC soon after miR-34a overexpression, nevertheless, the effect on Axl mRNA levels is much greater (Figure VD and VE in the [online-only Data Supplement](#)) indicating 2 different mechanisms of regulation. It has been demonstrated that miR-34a does not completely degrade SIRT1 transcript but interferes mainly with its translation.<sup>53,54</sup> In line with published evidences, during SMC and HASMC calcification, miR-34a decreases SIRT1 protein expression mainly by interfering with the translation of its transcript (Figures 4C, 4D, 4F, 6D, 6E, and 6G). On the contrary, miR-34a affects Axl protein expression by inducing degradation of the mRNA (Figures 4B, 4D, 4E, 6C, 6E, and 6F).

Taken together, our data suggest that miR-34a may promote VC by influencing proliferation and senescence of VSMC through different pathways; first, it inhibits cell growth partially via Axl downregulation and then enhances senescence by inhibiting SIRT1. Both events ultimately lead to VSMC osteochondrogenic transition (Graphic Abstract). Other miR-34a targets are likely to be involved in the calcification process: for instance, it has been reported that miR-34a can inhibit SMC proliferation via Notch1.<sup>55</sup>

A limitation of the present study is that soft tissue calcification has been induced in young animals. Future works must include aged *Mir34a*<sup>-/-</sup> and *Mir34a*<sup>+/+</sup> mice.

Altogether, the present study pinpoints miR-34a inhibition as a promising therapeutic approach for the treatment of age-related cardiovascular diseases.

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## Disclosures

None.

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### Highlights

- miR-34a genetic ablation reduces soft tissue and vascular calcification.
- miR-34a promotes vascular smooth muscle cell calcification by inducing cell growth arrest and senescence.
- miR-34a affects the expression of targets, such as Axl (AXL receptor tyrosine kinase) and SIRT1 (sirtuin 1), that are vascular calcification inhibitors.