# miR-181b regulates vascular endothelial aging by modulating a MAP3K3 signaling pathway

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## Abstract

Endothelial cell (EC) aging plays a vital role in the pathogenesis of cardiovascular disease (CVD). MicroRNAs have emerged as crucial regulators of target gene expression by inhibiting mRNA translation and/or promoting mRNA degradation. We identify an aging-related and oxidative stress-responsive microRNA, miR-181b, that inhibits endothelial cell apoptosis and senescence. In gain-or-loss function studies, miR-181b regulated the expression of key apoptosis markers (Bcl2, Bax, cleaved-Caspase3) and senescence markers (p16, p21, $\gamma$H2AX) and the ratio of apoptotic cells (TUNEL positive) and senescent cells (SA-$\beta$gal positive) in $H_2O_2$-induced ECs. Mechanistically, miR-181b targets MAP3K3 and modulates a MAP3K3/MKK/MAPK signaling pathway. MAP3K3 knockdown recapitulated the phenotype of miR-181b overexpression and miR-181b was dependent on MAP3K3 for regulating EC apoptosis and senescence. In vivo, miR-181b expression showed a negative correlation with increasing age in the mouse aorta. Endothelial-specific deficiency of miR-181a2b2 increased the target MAP3K3, markers of vascular senescence (p16, p21), and DNA double-strand breaks ($\gamma$H2AX) in the aorta of aged mice. Collectively, this study unveils an important role of miR-181b in regulating vascular endothelial aging via a MAP3K3-MAPK signaling pathway, providing new potential therapeutic targets for anti-aging therapy in CVD.

## Keywords

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## Introduction

Aging is a major risk factor for the development of cardiovascular disease, and people >65 years of age (especially >80 years of age) have a higher prevalence for all CVD[1]. Manifestation of disease is the culmination of the interaction between inherent genotypes and extrinsic factors, and aging is no exception[2]. Extrinsic factors can potently promote or postpone cellular and molecular cardiovascular aging and are regulated by therapeutic intervention, while genotype is a challenge to modify by current medical treatments[3]. Therefore, unveiling the mechanisms of extrinsic factors affecting aging and uncovering key nodes is valuable for anti-aging therapies. Oxidative stress, as an aging-accelerated extrinsic factor, plays an important role in the pathological process of vascular aging by producing excessive amounts of oxygen free radicals, which trigger senescence, and activate apoptotic signals in endothelial cells[4–6]. Apoptosis and senescence, as the key cellular response to environmental damages, are hallmarks of aging and are increasingly implicated in the aging process[7,8]. Oxidative stress induces cellular apoptosis in the intrinsic pathway by triggering the mitochondrial outer membrane permeabilization (MOMP), a tightly regulated event controlled by the Bcl2 family, including pro-apoptotic effector proteins, e.g. Bax and the antiapoptotic proteins, e.g. Bcl2[9,10]. Senescence includes replicative senescence (RS) and stress-induced premature senescence (SIPS). While the former naturally progresses over time, the latter is induced by extrinsic factors, e.g. oxidative stress[11]. Senescent cells can be identified by elevated senescence-associated β-galactosidase (SA-β-gal) activity, which is the most widely and easily used gold standard[12,13]. As terminally growth arrest appears in senescent cells, cell cycle regulators, e.g. p16 and p21, are also broadly employed biomarkers[14]. As a marker of DNA double-strand breaks and genomic instability in human population studies, γH2AX is also an important senescent biomarker[15].

MicroRNAs (miRNAs), are an emerging class of biomarkers and potential therapeutic targets for cardiovascular disease that negatively regulate gene expression by targeting specific mRNAs for cleavage or translational repression[16–18]. Accumulating studies highlight that miRNAs serve as key regulators in endothelial aging, often with an amplifying effect from the onset and progression of aging-related diseases. For example, miR-217, miR-34a, and miR-21 regulate endothelial aging by targeting SIRT1, a crucial controller in vascular aging[19–21]. miR-200c is induced by oxidative stress and regulates endothelial apoptosis and senescence via targeting ZEB1[22]. miR-181 family plays diverse roles in regulating key aspects of physiological and pathological processes in endothelial cells[23]. In our previous studies, systemic delivery of miR-181b demonstrated strong anti-atherosclerosis effects in mice[24]. Increasing aging is an independent risk factor for atherogenesis, and atherosclerosis per se is a disease of aging[25]. Therefore, identifying the potential effect of miR-181b in endothelial aging could open a new strategy to anti-aging therapies in CVD.

MAPK signal transduction pathways play a crucial role in regulating aging-related senescence and apoptosis[26]. MAPK cascades propel signal amplification and maintain signaling fidelity depending on a series of kinase proteins from MKKK (MAPK kinase kinase) through MKK (MAPK kinase) to a terminal MAPK such as p38, JNK, ERK1/2[27]. As a result of MAPK signal transduction, the expressions of aging markers (e.g. Bcl2, Bax,

p21) are regulated during the process of aging[28–30]. MAP3K3 (Mitogen-activated protein kinase kinase kinase 3), a key upstream regulator in the MAPK cascade, directly modulates phosphorylation of MKK3/MKK6 or MKK4, which then regulates substrates e.g. p38, JNK[31,32]. Some studies demonstrate key effects of MAP3K3 in apoptosis and senescence in diverse cell types[14,33–36].

In this study, we identify that miR-181b regulates endothelial aging by modulating the MAPK signaling pathway. We explore the mechanistic basis for miR-181b regulation of apoptosis and senescence in ECs that is dependent on a MAP3K3/MAPK signaling cascade. Finally, using EC-specific miR-181a2b2 knockout mice, we explore pre-mature aging markers for both senescence and apoptosis to reveal that this miR-181b-MAP3K3/MAPK signaling pathway may figure prominently in anti-aging strategies in CVD.

## Materials and Methods

### Animal Studies

MiR-181a2b2flox/flox (C57BL/6J) and miR-181a2b2 systemic knockout (miR-181a2b2$^{-/-}$) (C57BL/6J) mice were used as previously described[37] and tamoxifen-inducible endothelial-specific VE-cadherin (VECad-Cre-ERT2) (C57BL/6J) mice was kindly provided by R. Adams[38]. Inducible endothelial cell-specific miR-a2b2 deficient mice (MiR-181a2b2flox/flox; VECad-Cre-ERT2) was generated by crossbreeding miR-181a2b2flox/flox and VECad-Cre-ERT2 mice. For induction of Cre recombination, 4 weeks old male MiR-181a2b2flox/flox; VECad-Cre-ERT2 mice were treated with either 4-hydroxytamoxifen (H6278, Sigma) (10 mg/kg, i.p.) or same volume of corn oil vehicle for five consecutive days to generate ECs-specific miR-181a2b2 deficient mice (miR-181a2b2iECKO) and control mice (Ctrl). Male C57BL/6J mice at 20 weeks and 80 weeks old were purchased from Charles River Laboratory. Age- and cage-matched male littermates were used for experiments. All mice were maintained under SPF conditions at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the Brigham and Women's Hospital. All animal protocols were approved by the Institutional Animal Care and Use Committee at Brigham and Women's Hospital, Boston, MA and conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### Cell culture and transfection

HUVECs were purchased from Lonza (cc-2159) and cultured in EC growth medium EGM-2 (cc-3162, Lonza). HUVECs were plated on 12-well plates at 60, 000/well or 150, 000/well and allowed to grow to 80%−90% confluence under growing conditions or 70%−80% for transfection. Cells were stimulated with or without H2O2 (Sigma) at indicated dosage or camptothecin (CPT, 10 μM, C9911, Sigma) for various times, according to the experiment: Western blot, 2, 4, 8 or 16 hours; Real-time PCR, 1, 4 or 12 hours; Tunnel staining, 4 hours; senescence β-Galactosidase staining; 1 hour. Lipofectamine 2000 transfection reagent (11668019, Invitrogen) was used for transfection, following the manufacturer's instructions. MiRNA negative control (NS-m) (AM17110, Ambion) or pre-miR-181b (181b-m) (PM12442, Ambion) were transfected at 10 nM, and miRNA inhibitor negative control

(NS-i) (AM17010, Ambion) or miR-181b inhibitor (181b-i) (AM12442, Ambion) were transfected at 100 nM. SiRNA control (Ctrl si) (AM4636) and validated MEKK3 siRNA (MEKK3 si) (AM51331) from Invitrogen were transfected at 20 nM. For co-transfection studies, NS-i (100 nM), 181b-i (100 nM), Ctrl si (20 nM), or MEKK3 si (20 nM) were transfected as indicated in respective experiments.

### Luciferase activity assay

Putative miR-181b binding sites in the MEKK3 gene 3'UTR were predicted by the TargetScan algorithm. Individual wild-type or mutant binding site sequence was generated by annealing the forward and reverse oligonucleotides containing Xhol and Xbal sticky ends. The double-stranded oligonucleotides were ligated into the pcDNA3.1 (+) Luciferase vector, by using T4 DNA ligase. HUVECs were plated on 12-well plates at 60, 000/well up to 70%−80% confluency and transfected with 100 ng of wild-type or mutant MEKK3 reporter constructs and 10 ng Renilla plasmid (E2231, Promega). NS-m or 181b-m was co-transfected at 10 nM final concentration for 12 hours. Transfected cells were collected in 200 μl reporter lysis buffer (E1910, Promega) and luciferase activity was measured using a Dual-Luciferase reporter assay system (E1910, Promega). Each reading of luciferase activity was normalized to the Renilla activity.

### β-Galactosidase staining

β-Galactosidase activity assay was performed as previously described[39]. Briefly, HUVECs were transfected with and 24 hours post-transfection treated for 1 hours with $H_2O_2$ (30 μM) and cultured for another 3 days in normal growth medium before cells were fixed. β-Galactosidase activity at pH 6 was measured using X-Gal staining kit (9860, Cell Signaling).

### Immunofluorescence and Tunnel staining assay

For Immunofluorescence, 6 μm frozen sections of aortic roots were fixed and permeabilized with cold-acetone for 5 min and blocked in PBS containing 3% BSA for 1 hour at room temperature. Sections were stained with rat anti-CD31 (BD Pharmingen, 550274, 1:30), rabbit anti-p16 (1:100, ab51243, Abcam), rabbit anti-p21 (1:400, 2947, Cell Signaling), rabbit anti-γH2AX (2577, 1:800, Cell Signaling), cleaved caspase3 (1:400, 9661, Cell Signaling), Importin-α3 (1:100, ab84706, Abcam) and rabbit anti-MEKK3 (Cell Signaling) in PBS at 4 oC overnight. After three washes with PBS, the sections were further incubated with Alexa Flour 488-conjugated donkey anti-Rat IgG (Invitrogen, A21208, 1:300) and Alexa Flour 555-conjugated goat anti Rabbit IgG (Invitrogen, 1:300) in PBS for 1 hour at room temperature. Nuclei were stained for 5min at room temperature in PBS containing DAPI (Cell signaling, 4083, 0.5μg/ml). Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen). Images were acquired on an upright Carl Zeiss LSM 510 confocal. The data were calculated from at least 40 cells for each group with 6–10 mice. For each mouse, 2–5 plaques were selected and used for quantification. Tunnel protocol was performed as described by manufacturer's protocol (Millipore, ApopTaq Peroxidase Detection kit).

### RNA isolation and Real-time qPCR

Tissues were homogenized using TissueLyser II (Qiagen) according to manufacturer/s instructions. RNA isolation was using TRIzol Reagent according to the manufacturer's instructions. Subsequent RT-qPCR was performed using a High-Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems). For SyberGreen-based assay GoTaq qPCR Master Mix (A6001, Promega) was used, and for TaqMan Universal Master Mix II, UNG (4440038, Life Technologies) was used. Expression of mRNAs and miRNA expression were normalized to β-actin or U6 (Aglient, AriaMx Real Time PCR System). Specific primers including miR-181b-5p (#001098), U6 (#001973), human-HPRT (Hs05647472_cn), human-primary-miR-181b1 (Hs03302966_pri), human-primary-miR-181b2 (Hs03302963_pri), mouse-HPRT (Mm00522878_cn), mouse-primary-miR-181b1 (Mm03307120_pri) and mouse-primary-miR-181b2 (Mm03307414_pri) were purchased from Life Technologies. Changes in expression were calculated using delta delta Ct method.

### Western blot

Proteins were isolated using RIPA buffer (Boston BIoProductus, BP-115) with protease inhibitor and phosphatase inhibitor. Protein concentrations were determined using Pierce BCA kit (Thermo Scientific). 20 μg protein were loaded per lane on a 4–20% Mini-PROTEAN TGX Gel (Bio-Rad, 456–1096). Separated proteins were transferred to PVDF membranes using the Transfer Turbo Blot system (Bio-Rad) and Trans-Blot Turbo RTA Transfer Kit (Bio-Rad, 170–4272). The membrane was blocked with 5% nonfat milk in TBST for 1 hour at room temperature. After blocking, the membrane was incubated overnight at 4°C with antibodies against Bax (1:1000, 2772, Cell Signaling), Bcl-2 (1:1000, 3498, Cell Signaling), cleaved caspase-3 (1:1000, 9661, Cell Signaling), MEKK3 (1:1000, 5727, Cell Signaling), p16 (1:1000, ab51243, Abcam), p21(1:1000, ab109520, Abcam), p-$\gamma$H2AX (1:1000, 9718, Cell Signaling), p-MKK3/6 (1:1000, 12280, Cell Signaling), p-SEK1/MKK4 (1:1000, 4514, Cell Signaling), p-JNK (1:1000, 9255, Cell Signaling), p-p38 (1:1000, 8690, Cell Signaling), p-ERK1/2 (1:1000, 8544, Cell Signaling), p-IKB$\alpha$ (1:1000, 2859, Cell Signaling), JNK (1:1000, 9252, Cell Signaling), p38 (1:1000, 8690, Cell Signaling), ERK1/2 (1:1000, 4696, Cell Signaling), IKB$\alpha$ (1:1000, 4814, Cell Signaling), TAOK1 (1:1000, ab197891, Abcam) or β-actin (1:4000, 4970, Cell Signaling). Quantification of protein bands were performed using a luminescent image analyzer (BioRad, Chemidoc).

### Statistical analysis

GraphPad 7.0 software package (GraphPad Software, Inc) was used for statistical analysis. Unpaired two-tailed Student's t test was used to determine statistical significance between two groups for normally distributed continuous variables. For data without normal distribution, non-parametric Mann-Whitney U test was used. All data were expressed as mean ± SEM. $P < 0.05$ was considered significant for all tests.

## Results

### miR-181b is an oxidative stress-responsive microRNA reduced by aging.

miR-181b is a key regulator in multiple processes of pathology and physiology. In our previous studies, we found miR-181b exerted vasculo-protective effects in atherosclerosis, insulin resistance, and thrombosis[24,40,41]. Aging, as an independent risk factor, threatens vascular health and induces cardiovascular disease[42]. In order to investigate the role of miR-181b in vascular aging, we isolated RNA from the aorta of young (10 weeks old) and aged (80 weeks old) C57BL/6 mice for RT-qPCR (Fig.1 A). Compared to young mice, miR-181b expression of the aorta decreased by 40% in the aged mice. This decrease is in line with other stimuli known to reduce miR-181b expression including hyperlipidemia.[24,43] We further assessed the expression changes of miR-181b, the dominantly expressed miR-181 family member in endothelial cells[40], in $H_2O_2$-induced human umbilical vein endothelial cells (HUVECs). Expression of miR-181b was reduced in response to $H_2O_2$ in a dose- and time-dependent manner (Fig.1 B–C).

### miR-181b regulates apoptosis and senescence in ECs.

To assess the potential role of miR-181b in endothelial apoptosis and senescence, we examined the effect of miR-181b on aging-relevant phenotypes in $H_2O_2$–induced HUVECs by using gain- and loss-of-function experiments. Gain-of-function was induced by miR-181b mimics for miR-181b overexpression. Loss-of-function was induced by use of the complementary antagonist of miR-181b for miR-181b inhibition. We analyzed apoptosis markers, including Bax, Bcl-2, cleaved-Caspase3, and TUNEL staining, in $H_2O_2$–induced HUVECs. miR-181b overexpression reduced Bax, cleaved-Caspase3 protein expression by 17% and 54%, respectively, and increased Bcl-2 protein expression by 210% (Fig.2 A), while miR-181b inhibitors increased Bax and cleaved-Caspase3 protein expression by 129% and 127%, respectively, and reduced Bcl-2 protein expression by 45% (Fig.2 B). In TUNEL assays, miR-181b overexpression decreased apoptotic cells (TUNEL$^+$) by 25%, while miR-181b inhibitors increased it by 30% (Fig.2 C). For induction of senescence, $H_2O_2$ and CPT, an inhibitor of DNA topoisomerase I, can trigger DNA strand breaks[44]. In HUVECs stimulated by $H_2O_2$ or CPT, miR-181b overexpression reduced senescence markers, p16, p21, and γH2AX (a specific marker of DNA double-strand breaks) protein expression by 42%, 30%, and 41%, respectively (Fig.3 A). Using senescence-associated βgal (SA-βgal) as a marker of senescence in $H_2O_2$–induced HUVECs, we find that miR-181b overexpression decreased senescence cells (SA-βgal $^+$) by 50%, while miR-181b inhibitors increased it by 30% (Fig.3 B). Taken together, these findings indicate that miR-181b can inhibit apoptosis and senescence in $H_2O_2$–induced HUVECs.

### miR-181b regulates a MAPK signaling pathway.

To identify potential signaling pathways subject to miR-181b regulation of apoptosis and senescence, we overexpressed miR-181b using mimics. HUVECs were treated with $H_2O_2$ for 5, 15, 30, and 60 minutes, and we evaluated the phosphorylation of related signaling pathways. We found that miR-181b overexpression significantly reduced the phosphorylation of MKK3/MKK6 by 29%, and MKK4 by 36 % after 5, 15 minutes, respectively (Fig.4 A). Furthermore, we investigated the phosphorylation of JNK or p38,

which are the downstream substrates of MKK3/MKK6 or MKK4, that directly regulate transcription factors to affect gene expression[45]. In $H_2O_2$-induced HUVECs, miR-181b overexpression significantly reduced the phosphorylation of p38 by 48%, while JNK, ERK1/2 was not impacted (Fig.4 A, Supplementary Figure 1). Interestingly, in $H_2O_2$-induced HUVECs, miR-181b had no effect on the NF-κB signaling, a pathway that miR-181b markedly repressed in response to TNF-α or LPS[46] (Supplementary Figure 1). Collectively, these data indicated that in response to $H_2O_2,$ miR-181b inhibited the MAPK signaling pathway.

**miR-181b targets the expression of MAP3K3.**

Using algorithms of TargetScan, miRDB, and miRWalk, predicted targets of miR-181b were identified (Fig.5 A). Aligning these potential targets with those known to be involved in MAPK signaling pathways revealed two targets of interest, MAP3K3 and TAOK1. Overexpression of miR-181b did not affect the protein expression of TAOK1 or phosphorylated TAOK1 (Supplementary Figure 2). However, in miR-181b–overexpressing HUVECs, mRNA expression of MAP3K3 was reduced by 40% (Fig.5 B), and protein expression of MAP3K3 was reduced by 50% (Fig.5 C). In contrast, miR-181b inhibition in HUVECs increased MAP3K3 mRNA expression by 50% (Fig.5 D) and protein expression by 40% (Fig.5 E), suggesting that miR-181b is a negative regulator of MAP3K3. Analysis of miR-181b–binding sites in the 3'-UTR of MAP3K3 by TargetScan revealed conserved sites across species (Fig.5 F). To investigate whether the miR-181b binding site is functionally active in the MAP3K3 3'-UTR, we generated a luciferase-reporter construct harboring wildtype and mutant miR-181b binding sites (Fig.5 G). MiR-181b overexpression decreased luciferase activity by 25% in luciferase-reporter constructs containing wildtype 3'UTR of MAP3K3, while constructs that contained the mutated 3'-UTR was not affected (Fig.5 H). Taken together, these findings indicate that miR-181b targets the 3'UTR of MAP3K3 to repress its mRNA and protein expression. Because MAP3K3, is known as a key upstream regulator in the MAPK signaling cascade, and modulates signal transduction by directly regulating phosphorylation of MKK3/MKK6, MKK4[31,32], these findings are in accordance with the signaling pathway modulation observed by miR-181b (Fig.4 A).

**MAP3K3 regulates endothelial apoptosis and senescence by modulating the MAPK signaling pathway.**

To investigate whether MAP3K3 deficiency can reproduce the inhibitory effects of miR-181b by modulating the MAPK signaling pathway, we performed siRNA-mediated knockdown studies in HUVECs[47]. For apoptosis, MAP3K3 knockdown reduced protein expression of Bax and cleaved-Caspase3 by 16% and 21%, respectively, and increased Bcl-2 by 174% (Fig.6 A). MAP3K3 knockdown also decreased apoptotic cells (TUNEL+) by 25% in $H_2O_2$–induced HUVECs (Fig.6 B) and senescence cells (SA-βgal +) by 50% (Fig.6 C). Moreover, we evaluated if MAP3K3, as a key upstream regulator in the MAPK signaling cascade, modulated the MAPK signaling pathway in $H_2O_2$–induced HUVECs. Indeed, MAP3K3 knockdown significantly reduced the phosphorylation of MAP2Ks, (e.g., MKK3/MKK6, MKK4), and MAPKs, (e.g., ERK, p38) (Fig.6 D). Taken together, siRNA-mediated knockdown of MAP3K3 recapitulates the effects of miR-181b on apoptosis and senescence by modulating the MAPK signaling pathway in $H_2O_2$–induced HUVECs.

**miR-181b is dependent on MAP3K3 for regulating apoptosis and senescence in ECs.**

To test whether the miR-181b-mediated effect on apoptosis and senescence is dependent on MAP3K3, we used miR-181b inhibitors or mimics with simultaneous MAP3K3 siRNA knockdown. In HUVECs stimulated with $H_2O_2$, MAP3K3 knockdown reversed the increased expression of Bax or cleaved-Caspase3 and the reduction of Bcl-2 mediated by miR-181b inhibitors at the protein level (Fig.7 A). Furthermore, MAP3K3 silencing neutralized the increase of apoptotic cells (TUNEL+) induced by miR-181b inhibitors (Fig.7 B). In SA-βgal staining of $H_2O_2$–induced HUVECs, MAP3K3 knockdown counteracted the increase in senescence cells (SA-βgal +) induced by miR-181b inhibitors (Fig.7 C). Finally, silencing of MAP3K3 reinforced senescence induced by miR-181b overexpression (Fig.7 D).

**miR-181b deficiency induces aging *in vivo*.**

To investigate the effect of miR-181b on aging *in vivo*, we first isolated the RNA of different organs from systemic miR-181a2b2 knockout mice to examine the global effect of miR-181b on apoptosis and senescence. We found the mRNA expression of p21 was induced by systemic miR-181a2b2 knockout in several organs, while other markers of apoptosis or senescence were unchanged (Supplementary Figure 3). To assess the effects on apoptosis and senescence in ECs, we recently generated the endothelial-specific miR-181a2b2 knockout (EC-miR-181a2b2 KO) mice to further explore the *in vivo* phenotype of miR-181b in the vascular endothelium.[43] These EC-miR-181a2b2 KO mice harbor a marked reduction in miR-181b expression in the aortic intima, whereas there was minimal change in miR-181a likely due to its much lower relative expression.[43] Immunofluorescence staining results showed markedly increased positive cells of p21, p16, γH2AX, and MAP3K3 in the aorta sinus of endothelial-specific miR-181a2b2 knockout mice by 18 months (Fig.8) compared to controls. Taken together, these findings reveal that deficiency of miR-181a2b2 triggers hallmarks of vascular endothelial senescence *in vivo*.

## Discussion

Aging is among the strongest risk factors that threatens health and contributes to organ dysfunction. Cellular aging ultimately leads to organismal aging[48]. In the cardiovascular system, endothelial aging alters endothelial homeostasis triggering atherosclerosis, coronary heart disease, stroke, and a range of other ischemic cardiovascular diseases[42,49]. A better understanding of key drivers of endothelial aging may provide insights to promote reparative cell and organismal function. In our study, we identified miR-181b as a key regulator in endothelial aging by targeting MAP3K3, an upstream regulator of MAPK signaling. In mice, miR-181b expression was inversely correlated with increasing age (Fig.1 A).

In the biological process of cells, miR-181b regulates a diverse array of cellular functions and maintains cellular homeostasis by controlling cell death and cellular senescence, which are two closely related pathways in aging. In our previous studies, miR-181b, as an inflammation-responsive microRNA, inhibited ECs inflammation, which protected vessels from atherosclerosis in ApoE–deficient mice[24]. Apoptosis is an aging-related programmed cell death. In different cell types, miR-181b broadly exerts inhibition on apoptosis, such

as in hypoxia-evoked H9c2 cells[50], hypertrophic scar fibroblasts[51], AML cells[52], and SW480 cells[53]. However, to date there has not been a definitive study to illustrate the effect of miR-181b on endothelial apoptosis. In this study, we identified the anti-apoptosis phenotype of miR-181b in $H_2O_2$–induced ECs by gain- or loss-of-function. The intrinsic pathway takes a dominant role in oxidative stress-induced apoptosis. Indeed, miR-181b decreased Bax, a proapoptotic effector protein, and increased Bcl2, an antiapoptotic protein, in response to $H_2O_2$-mediated oxidative stress (Fig.2 A, B). As the shared key mediator of apoptosis by both intrinsic and extrinsic pathways, caspase3 is cleaved at an aspartate residue to yield cleaved caspase-3, which then initiates cellular apoptosis. MiR-181b overexpression decreased, while miR-181b inhibition increased the protein expression of cleaved caspase3 in $H_2O_2$–induced ECs (Fig.2 A, B). Moreover, TUNEL positive cells were also significantly reduced by miR-181b mimics, while increased by miR-181b inhibitor (Fig.2 C). Collectively, these findings suggest that miR-181b plays a vital role in the regulation of endothelial apoptosis.

In cancer biology, the effect of miR-181b on apoptosis is complicated[54]. In human gastric adenocarcinoma cells and human lung cancer cells, miR-181b induced Bcl-2 expression and protected cells from apoptosis[55]. miR-181b overexpression induced Bcl-2 and reduced Bax and cleaved caspase3 expression, which decreased the proportion of apoptotic human colon cancer cells[56]. In human cervical cancer cells, prostate cancer cells, and thyroid papillary cancer cells, miR-181b also showed the anti-apoptotic effect[57–59]. However, in chronic lymphocytic leukemia and astrocytoma, miR-181b induced apoptosis[60,61]. In different non-tumor cell types, miR-181b usually exerts inhibition on apoptosis. miR-181b-5p inhibited fibroblast apoptosis through MEK/ERK/p21 pathway[62]. Cardiomyocyte apoptosis is inhibited by miR-181b via directly targeting HMGB1[63]. miR-181b suppressed mesangial apoptosis by regulating TIMP3[64]. In human renal proximal tubule cells, miR-181b inhibitor promoted apoptosis and miR-181b overexpression attenuated apoptosis induced by MEG3 overexpression[65]. Finally, by interacting with NEAT1, inhibition of miR-181b induced apoptosis in hypoxia-evoked cardiomyoblasts via PI3K/AKT/mTOR and JAK1/STAT3 pathways[50]. Taken together, these data indicate that miR-181b can exhibit either anti-apoptotic or pro-apoptotic effects in different cancer cell types, whereas in primary cells miR-181b generally exerts anti-apoptotic effects.

Cellular senescence, a stable form of cell cycle arrest, is typically induced in response to diverse stressors[66,67]. Hallmarks of senescent cells include DNA damage, expression of cyclin-dependent kinase inhibitors and senescence-associated β-galactosidase (SA-β-gal), induction of the senescence-associated secretory phenotype, alterations in chromatin remodeling, and metabolism[68,69]. In support of a role of miR-181b in endothelial senescence, we found that miR-181b expression decreased in the aorta of aged mice compared to young mice (Fig.1 A), and fell in response to $H_2O_2$ treatment in endothelial cells (Fig.1 B, C), which hinted at its potential regulation in the endothelial senescence. In ECs stimulated by $H_2O_2$ or CPT (which induces DNA double-strand breaks), miR-181b overexpression reduced the cyclin dependent kinase inhibitor senescent markers, p16, p21 and γH2AX, a definitive marker for DNA double-strand breaks[70] (Fig.3 A). In the SA-βgal staining of $H_2O_2$–induced ECs, miR-181b overexpression potently reduced the proportion of senescent ECs, while miR-181b inhibition induced it (Fig.3 B). To unveil the

regulation of miR-181b on apoptosis and senescence in the vascular endothelium *in vivo*, we utilized endothelial-specific miR-181a2b2 knockout mice, which showed robust expression of senescent markers p16, p21, and $\gamma$H2AX in aortic endothelium (Fig.8). Because there was a potent decrease in p21 expression in aortas and several other organs in the systemic miR-181a2b2 knockout (Supplementary Figure 3), it is likely that other cell types in addition to endothelial cells contribute to cellular senescence in this mouse model. We recently found that these mice develop accelerated atherosclerotic lesion formation in the aorta when placed on a Western diet in the presence of AAV-PCSK9 gain-of-function mutation overexpression.[43] While we have not performed formal longitudinal studies that would be required to address the life span of these mice, the constellation of aging markers in the vascular endothelium (Fig. 8) including $\gamma$H2AX for DNA double strand breaks, p16 and p21 for cellular senescence, and MAP3K3 for inflammatory signaling constitute some of the key features found in several aging mouse models.[71] Collectively, the miR-181b-mediated regulation of apoptosis and senescence indicates that miR-181b plays an important role in endothelial aging.

As an essential upstream regulator in the MAPK cascade, MAP3K3 regulates apoptosis and senescence. MAP3K3 knockdown blocked mitochondrial impairment by inhibiting the loss of mitochondrial membrane potential and cytochrome C expression as well as promoting ATP synthesis and reduced apoptosis and mitochondrial damage in cardiomyocytes in response to hypoxia/reoxygenation (H/R)[34]. MAP3K3 deficiency also increased the lifespan in *C.elegans* and decreased the levels of ROS[72]. In the current study, we also found that MAP3K3 knockdown reduced apoptosis and senescence markers in ECs responding to $H_2O_2$ through the MAPK signaling pathway (Fig.6 A–D). Using a combination of prediction algorithms and alignment with potential MAPK-associated targets, we verified that MAP3K3 is a target of miR-181b in ECs. Importantly, miR-181b was dependent on MAP3K3 to regulate EC apoptosis and senescence (Fig.7 A–D). Given the known cross-talk between NF-kB and MAPK signaling pathways, it is possible that the reduction in miR-181b expression in response to senescent stimuli may be mediated in part by NF-$\kappa$B site. In support, we identified several putative NF-$\kappa$B binding sites in the promoter region of MIR181B2 (Supplementary Figure 4), the dominantly expressed miR-181b isoform in endothelial cells.[24,40] Future studies will be of interest to further delineate this potential feedback loop. Taken together, these findings underscore a prominent role for miR-181b in regulating apoptosis and senescence in ECs via a MAP3K3/MAPK pathway.

In summary, we identified miR-181b as an aging-related and oxidative stress-responsive microRNA that inhibits endothelial cell apoptosis and senescence. MiR-181b expression inversely correlated with increasing age in the aorta and was repressed by $H_2O_2$-treated ECs. Gain or loss-of-function studies revealed that miR-181b regulated the expression of key apoptosis (Bcl2, Bax, cleaved-Caspase3) and senescence markers (p16, p21, $\gamma$H2AX) in $H_2O_2$-induced ECs. On the molecular level, miR-181b targets MAP3K3, a known upstream regulator of MAPK signaling. MiR-181b was dependent on MAP3K3 for regulating EC apoptosis and senescence, and MAP3K3 knockdown recapitulated the phenotype of miR-181b overexpression. EC-miR-181a2b2 KO mice showed increased expression of the target MAP3K3, markers of vascular senescence (p16, p21), and DNA double-strand breaks ($\gamma$H2AX) in the aorta of aged mice. These findings establish an important role of miR-181b

in regulating vascular endothelial aging via a MAP3K3-MAPK signaling pathway, providing new insights for anti-aging therapies in the vascular endothelium.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

## Non-standard Abbreviations and Acronyms

| | |
|---|---|
| **BMDM** | Bone marrow derived macrophages |
| **CVD** | Cardiovascular disease |
| **EC** | Endothelial cell |
| **HCD** | High cholesterol diet |
| **IHC** | Immunohistochemistry |
| **MAPK** | Mitogen-activated protein kinases |
| **PBMC** | Peripheral blood mononuclear cell |
| **RT-qPCR** | Real-time polymerase chain reaction |
| **TUNEL** | Terminal deoxynucleotidyl transferase dUTP nick end labeling |

## References

1. Heidenreich PA et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. Circulation 123, 933–944, doi:10.1161/CIR.0b013e31820a55f5 (2011). [PubMed: 21262990]

2. Anton B, Vitetta L, Cortizo F & Sali A Can we delay aging? The biology and science of aging. Ann N Y Acad Sci 1057, 525–535 (2005). [PubMed: 16399917]

3. Gude NA, Broughton KM, Firouzi F & Sussman MA Cardiac ageing: extrinsic and intrinsic factors in cellular renewal and senescence. Nat Rev Cardiol 15, 523–542, doi:10.1038/s41569-018-0061-5 (2018). [PubMed: 30054574]

4. Munzel T et al. Impact of Oxidative Stress on the Heart and Vasculature: Part 2 of a 3-Part Series. J Am Coll Cardiol 70, 212–229, doi:10.1016/j.jacc.2017.05.035 (2017). [PubMed: 28683969]

5. Donato AJ, Machin DR & Lesniewski LA Mechanisms of Dysfunction in the Aging Vasculature and Role in Age-Related Disease. Circ Res 123, 825–848, doi:10.1161/CIRCRESAHA.118.312563 (2018). [PubMed: 30355078]

6. Aoki M et al. Endothelial apoptosis induced by oxidative stress through activation of NF-kappaB: antiapoptotic effect of antioxidant agents on endothelial cells. Hypertension 38, 48–55 (2001). [PubMed: 11463759]

7. Tower J Programmed cell death in aging. Ageing Res Rev 23, doi:10.1016/j.arr.2015.04.002 (2015).

8. McHugh D & Gil J Senescence and aging: Causes, consequences, and therapeutic avenues. J Cell Biol 217, 65–77, doi:10.1083/jcb.201708092 (2018). [PubMed: 29114066]

9. Green DR & Reed JC Mitochondria and apoptosis. Science 281, 1309–1312, doi:10.1126/science.281.5381.1309 (1998). [PubMed: 9721092]

10. Green DR & Llambi F Cell Death Signaling. Cold Spring Harb Perspect Biol 7, doi:10.1101/cshperspect.a006080 (2015).

11. Sikora E, Arendt T, Bennett M & Narita M Impact of cellular senescence signature on ageing research. Ageing Res Rev 10, 146–152, doi:10.1016/j.arr.2010.10.002 (2011). [PubMed: 20946972]

12. Debacq-Chainiaux F, Erusalimsky JD, Campisi J & Toussaint O Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc 4, 1798–1806, doi:10.1038/nprot.2009.191 (2009). [PubMed: 20010931]

13. Dimri GP et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92, 9363–9367 (1995). [PubMed: 7568133]

14. Collado M & Serrano M Senescence in tumours: evidence from mice and humans. Nat Rev Cancer 10, 51–57, doi:10.1038/nrc2772 (2010). [PubMed: 20029423]

15. Valdiglesias V, Giunta S, Fenech M, Neri M & Bonassi S gammaH2AX as a marker of DNA double strand breaks and genomic instability in human population studies. Mutat Res 753, 24–40, doi:10.1016/j.mrrev.2013.02.001 (2013). [PubMed: 23416207]

16. Condorelli G, Latronico MV & Cavarretta E microRNAs in cardiovascular diseases: current knowledge and the road ahead. J Am Coll Cardiol 63, 2177–2187, doi:10.1016/j.jacc.2014.01.050 (2014). [PubMed: 24583309]

17. Dangwal S & Thum T microRNA therapeutics in cardiovascular disease models. Annu Rev Pharmacol Toxicol 54, 185–203, doi:10.1146/annurev-pharmtox-011613-135957 (2014). [PubMed: 24111539]

18. Bartel DP MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233, doi:10.1016/j.cell.2009.01.002 (2009). [PubMed: 19167326]

19. Menghini R et al. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation 120, 1524–1532, doi:10.1161/CIRCULATIONAHA.109.864629 (2009). [PubMed: 19786632]

20. Ito T, Yagi S & Yamakuchi M MicroRNA-34a regulation of endothelial senescence. Biochem Biophys Res Commun 398, 735–740, doi:10.1016/j.bbrc.2010.07.012 (2010). [PubMed: 20627091]

21. Guo Y et al. Kallistatin reduces vascular senescence and aging by regulating microRNA-34a-SIRT1 pathway. Aging Cell 16, 837–846, doi:10.1111/acel.12615 (2017). [PubMed: 28544111]

22. Magenta A et al. miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death Differ 18, 1628–1639, doi:10.1038/cdd.2011.42 (2011). [PubMed: 21527937]

23. Sun X, Sit A & Feinberg MW Role of miR-181 family in regulating vascular inflammation and immunity. Trends Cardiovasc Med 24, 105–112, doi:10.1016/j.tcm.2013.09.002 (2014). [PubMed: 24183793]

24. Sun X et al. Systemic delivery of microRNA-181b inhibits nuclear factor-kappaB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice. Circ Res 114, 32–40, doi:10.1161/CIRCRESAHA.113.302089 (2014). [PubMed: 24084690]

25. Wang JC & Bennett M Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. Circ Res 111, 245–259, doi:10.1161/CIRCRESAHA.111.261388 (2012). [PubMed: 22773427]

26. Pearson G et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22, 153–183, doi:10.1210/edrv.22.2.0428 (2001). [PubMed: 11294822]

27. Raman M, Chen W & Cobb MH Differential regulation and properties of MAPKs. Oncogene 26, 3100–3112, doi:10.1038/sj.onc.1210392 (2007). [PubMed: 17496909]

28. Liu YZ, Boxer LM & Latchman DS Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade. Nucleic Acids Res 27, 2086–2090, doi:10.1093/nar/27.10.2086 (1999). [PubMed: 10219080]

29. Papadakis ES et al. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. FEBS Lett 580, 1320–1326, doi:10.1016/j.febslet.2006.01.053 (2006). [PubMed: 16458303]

30. Beier F, Taylor AC & LuValle P The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. J Biol Chem 274, 30273–30279, doi:10.1074/jbc.274.42.30273 (1999). [PubMed: 10514521]

31. Deacon K & Blank JL MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. The Journal of biological chemistry 274, 16604–16610 (1999). [PubMed: 10347227]

32. Deacon K & Blank JL Characterization of the mitogen-activated protein kinase kinase 4 (MKK4)/c-Jun NH2-terminal kinase 1 and MKK3/p38 pathways regulated by MEK kinases 2 and 3. MEK kinase 3 activates MKK3 but does not cause activation of p38 kinase in vivo. The Journal of biological chemistry 272, 14489–14496 (1997). [PubMed: 9162092]

33. Guo SY, Liu SG, Liu L, Zhou XJ & Gu Y RNAi silencing of the MEKK3 gene promotes TRAIL-induced apoptosis in MCF-7 cells and suppresses the transcriptional activity of NF-kappaB. Oncol Rep 27, 441–446, doi:10.3892/or.2011.1509 (2012). [PubMed: 22020406]

34. Wang L, Yan W & Wang J Silencing MEKK3 attenuates cardiomyocyte injury caused by hypoxia/reoxygenation via the sonic hedgehog pathway. J Cell Physiol, doi:10.1002/jcp.28162 (2019).

35. Xu LG, Li LY & Shu HB TRAF7 potentiates MEKK3-induced AP1 and CHOP activation and induces apoptosis. J Biol Chem 279, 17278–17282, doi:10.1074/jbc.C400063200 (2004). [PubMed: 15001576]

36. Ellinger-Ziegelbauer H, Kelly K & Siebenlist U Cell cycle arrest and reversion of Ras-induced transformation by a conditionally activated form of mitogen-activated protein kinase kinase kinase 3. Mol Cell Biol 19, 3857–3868 (1999). [PubMed: 10207109]

37. Henao-Mejia J et al. The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. Immunity 38, 984–997, doi:10.1016/j.immuni.2013.02.021 (2013). [PubMed: 23623381]

38. Wang Y et al. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465, 483–486, doi:10.1038/nature09002 (2010). [PubMed: 20445537]

39. Haemmig S et al. Long noncoding RNA SNHG12 integrates a DNA-PK-mediated DNA damage response and vascular senescence. Science translational medicine 12, doi:10.1126/scitranslmed.aaw1868 (2020).

40. Sun X et al. MicroRNA-181b regulates NF-κB-mediated vascular inflammation. J Clin Invest 122, 1973–1990, doi:10.1172/JCI61495 (2012). [PubMed: 22622040]

41. Lin J et al. MicroRNA-181b inhibits thrombin-mediated endothelial activation and arterial thrombosis by targeting caspase recruitment domain family member 10. FASEB J 30, 3216–3226, doi:10.1096/fj.201500163R (2016). [PubMed: 27297585]

42. Paneni F, Diaz Canestro C, Libby P, Luscher TF & Camici GG The Aging Cardiovascular System: Understanding It at the Cellular and Clinical Levels. J Am Coll Cardiol 69, 1952–1967, doi:10.1016/j.jacc.2017.01.064 (2017). [PubMed: 28408026]

43. Yang DH,S; Chen J; McCoy MG; Cheng HS; Zhou H; Pérez-Cremades D; Cheng X; Sun X; Haneo-Mejia J; Vellarikkal SK; Gupta RM; Barrera V; Feinberg MW Endothelial cell-specific deletion of a microRNA accelerates atherosclerosis. Atherosclerosis, doi:DOI:10.1016/j.atherosclerosis.2022.04.010 (2022).

44. Pommier Y Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer 6, 789–802, doi:10.1038/nrc1977 (2006). [PubMed: 16990856]

45. Dérijard B et al. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science (New York, N.Y.) 267, 682–685 (1995). [PubMed: 7839144]

46. Sun X et al. MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. J Clin Invest 122, 1973–1990, doi:10.1172/JCI61495 (2012). [PubMed: 22622040]

47. Lam JK, Chow MY, Zhang Y & Leung SW siRNA Versus miRNA as Therapeutics for Gene Silencing. Mol Ther Nucleic Acids 4, e252, doi:10.1038/mtna.2015.23 (2015). [PubMed: 26372022]

48. Jeyapalan JC & Sedivy JM Cellular senescence and organismal aging. Mech Ageing Dev 129, 467–474, doi:10.1016/j.mad.2008.04.001 (2008). [PubMed: 18502472]

49. Ungvari Z et al. Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. Nat Rev Cardiol 15, 555–565, doi:10.1038/s41569-018-0030-z (2018). [PubMed: 29795441]

50. Lv Y et al. LncRNA nuclear-enriched abundant transcript 1 regulates hypoxia-evoked apoptosis and autophagy via mediation of microRNA-181b. Mol Cell Biochem 464, 193–203, doi:10.1007/s11010-019-03660-2 (2020). [PubMed: 31853799]

51. Liu B, Guo Z & Gao W miR-181b-5p promotes proliferation and inhibits apoptosis of hypertrophic scar fibroblasts through regulating the MEK/ERK/p21 pathway. Exp Ther Med 17, 1537–1544, doi:10.3892/etm.2019.7159 (2019). [PubMed: 30783419]

52. Chen H, Chen Q, Fang M & Mi Y microRNA-181b targets MLK2 in HL-60 cells. Sci China Life Sci 53, 101–106, doi:10.1007/s11427-010-0002-y (2010). [PubMed: 20596961]

53. Liu Y et al. miR-181b functions as an oncomiR in colorectal cancer by targeting PDCD4. Protein Cell 7, 722–734, doi:10.1007/s13238-016-0313-2 (2016). [PubMed: 27647131]

54. Liu J, Shi W, Wu C, Ju J & Jiang J miR-181b as a key regulator of the oncogenic process and its clinical implications in cancer (Review). Biomed Rep 2, 7–11, doi:10.3892/br.2013.199 (2014). [PubMed: 24649060]

55. Zhu W, Shan X, Wang T, Shu Y & Liu P miR-181b modulates multidrug resistance by targeting BCL2 in human cancer cell lines. Int J Cancer 127, 2520–2529, doi:10.1002/ijc.25260 (2010). [PubMed: 20162574]

56. Yang X, Sun Y, Zhang Y & Han S Downregulation of miR181b inhibits human colon cancer cell proliferation by targeting CYLD and inhibiting the NFkappaB signaling pathway. Int J Mol Med 46, 1755–1764, doi:10.3892/ijmm.2020.4720 (2020). [PubMed: 32901872]

57. Yang L et al. miR-181b promotes cell proliferation and reduces apoptosis by repressing the expression of adenylyl cyclase 9 (AC9) in cervical cancer cells. FEBS Lett 588, 124–130, doi:10.1016/j.febslet.2013.11.019 (2014). [PubMed: 24269684]

58. He L et al. MicroRNA-181b expression in prostate cancer tissues and its influence on the biological behavior of the prostate cancer cell line PC-3. Genet Mol Res 12, 1012–1021, doi:10.4238/2013.April.2.17 (2013). [PubMed: 23613247]

59. Li D et al. Down-regulation of miR-181b promotes apoptosis by targeting CYLD in thyroid papillary cancer. Int J Clin Exp Pathol 7, 7672–7680 (2014). [PubMed: 25550803]

60. Zhu DX et al. miR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes. Carcinogenesis 33, 1294–1301, doi:10.1093/carcin/bgs179 (2012). [PubMed: 22610076]

61. Zhi F et al. MiR-181b-5p downregulates NOVA1 to suppress proliferation, migration and invasion and promote apoptosis in astrocytoma. PLoS One 9, e109124, doi:10.1371/journal.pone.0109124 (2014). [PubMed: 25299073]

62. Liu B, Guo Z & Gao W miR-181b-5p promotes proliferation and inhibits apoptosis of hypertrophic scar fibroblasts through regulating the MEK/ERK/p21 pathway. Exp Ther Med 17, 1537–1544, doi:10.3892/etm.2019.7159 (2019). [PubMed: 30783419]

63. Ling L, Zhi L, Wang H, Deng Y & Gu C MicroRNA-181b Inhibits Inflammatory Response and Reduces Myocardial Injury in Sepsis by Downregulating HMGB1. Inflammation 44, 1263–1273, doi:10.1007/s10753-020-01411-w (2021). [PubMed: 34076811]

64. Zhu FX, Wu HL, Chen JX, Han B & Guo YF Dysregulation of microRNA-181b and TIMP3 is functionally involved in the pathogenesis of diabetic nephropathy. J Cell Physiol 234, 18963–18969, doi:10.1002/jcp.28536 (2019). [PubMed: 30937907]

65. Pang X et al. Inhibition of lncRNA MEG3 protects renal tubular from hypoxia-induced kidney injury in acute renal allografts by regulating miR-181b/TNF-alpha signaling pathway. J Cell Biochem 120, 12822–12831, doi:10.1002/jcb.28553 (2019). [PubMed: 30860638]

66. Hernandez-Segura A, Nehme J & Demaria M Hallmarks of Cellular Senescence. Trends Cell Biol 28, 436–453, doi:10.1016/j.tcb.2018.02.001 (2018). [PubMed: 29477613]

67. Munoz-Espin D & Serrano M Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol 15, 482–496, doi:10.1038/nrm3823 (2014). [PubMed: 24954210]

68. Herranz N & Gil J Mechanisms and functions of cellular senescence. J Clin Invest 128, 1238–1246, doi:10.1172/JCI95148 (2018). [PubMed: 29608137]

69. Gorgoulis V et al. Cellular Senescence: Defining a Path Forward. Cell 179, 813–827, doi:10.1016/j.cell.2019.10.005 (2019). [PubMed: 31675495]

70. Kuo LJ & Yang LX Gamma-H2AX - a novel biomarker for DNA double-strand breaks. In Vivo 22, 305–309 (2008). [PubMed: 18610740]

71. Koks S et al. Mouse models of ageing and their relevance to disease. Mech Ageing Dev 160, 41–53, doi:10.1016/j.mad.2016.10.001 (2016). [PubMed: 27717883]

72. Chamoli M, Singh A, Malik Y & Mukhopadhyay A A novel kinase regulates dietary restriction-mediated longevity in Caenorhabditis elegans. Aging Cell 13, 641–655, doi:10.1111/acel.12218 (2014). [PubMed: 24655420]
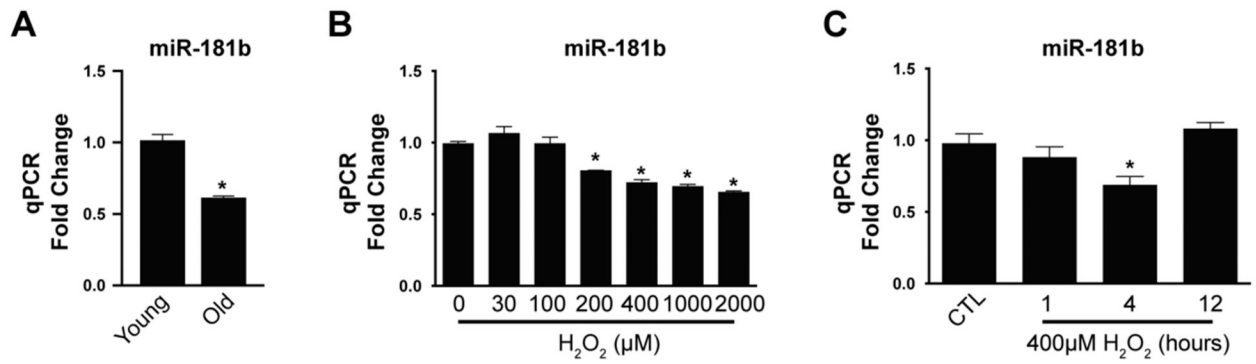
**Fig.1. miR-181b expression is regulated by aging.**
**(A)** miR-181b expression of the aorta between young (10w) and old mice (80w) (n=4). **(B)** Expression of miR-181b in response to different concentrations of $H_2O_2$ (n=4). **(C)** Kinetics of miR-181b in ECs stimulated with $H_2O_2$ (n=4).
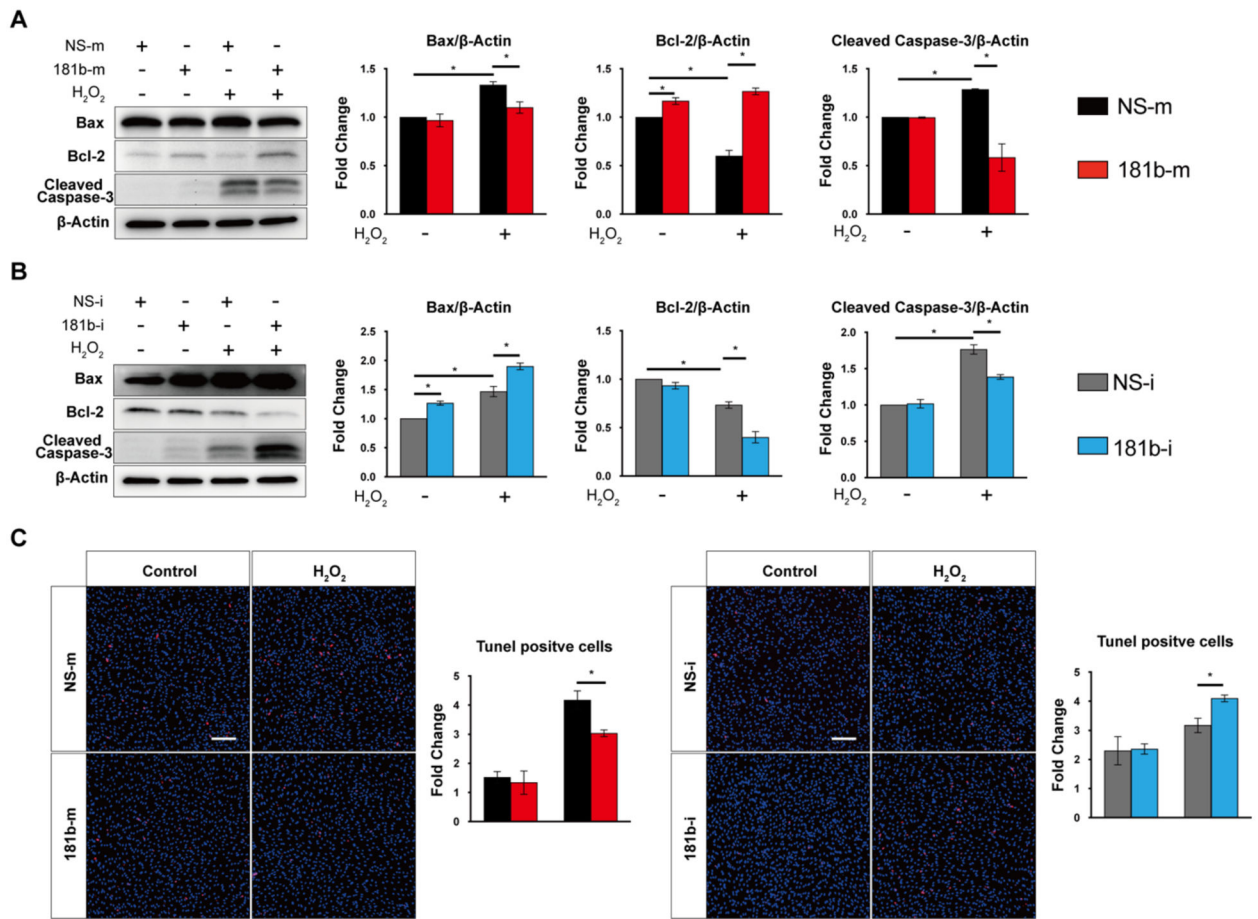
**Fig.2. miR-181b regulation of endothelial apoptosis.**
miR-181b mimics (**A**) and inhibitors (**B**) regulate apoptotic markers (n=3). (**C**) TUNEL staining of HUVEC transfected with miR-181b mimics (n=4–5) or miR-181b inhibitors (n=4–5).
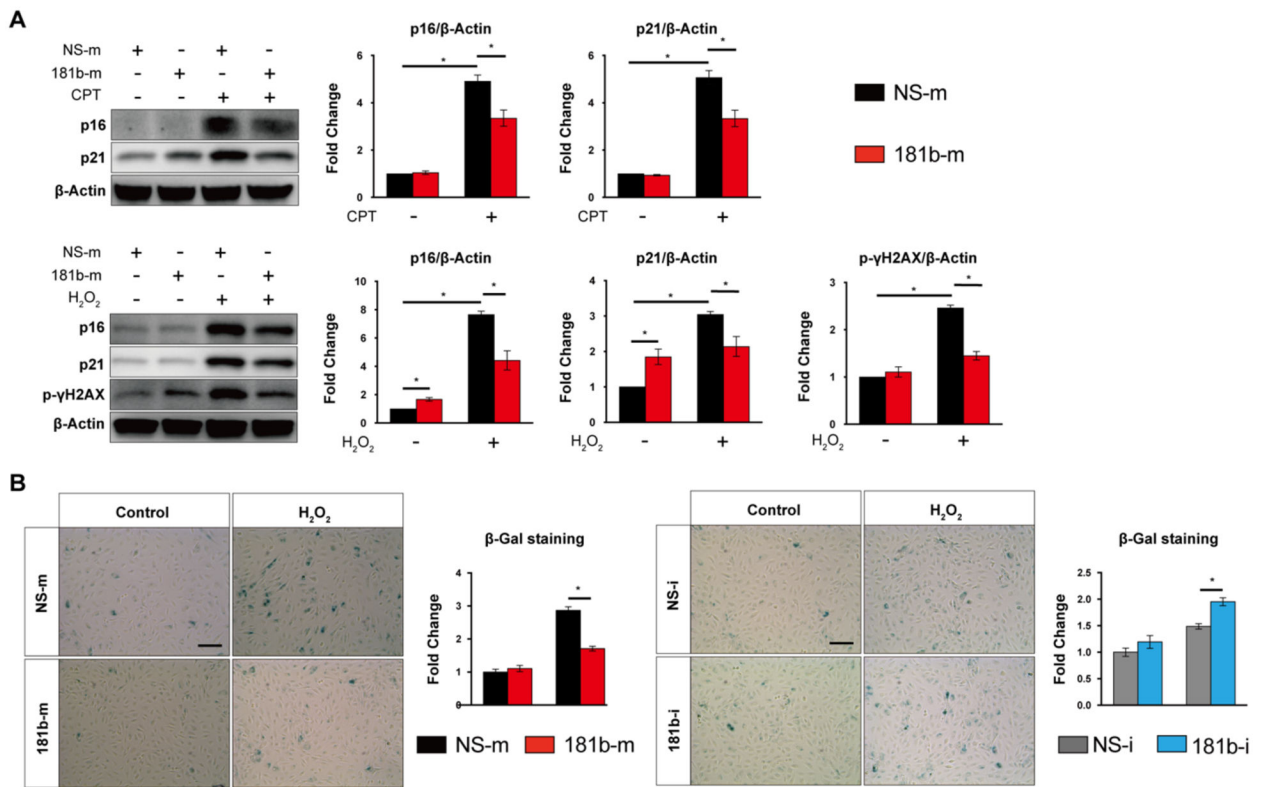
**Fig.3. miR-181b regulation of endothelial senescence.**
**(A)** miR-181b mimics regulate senescence markers with stimuli of CPT or H$_2$O$_2$ (n=3).
**(B)** SA-βgal staining of HUVEC transfected with miR-181b mimics (n=4–6) or miR-181b
inhibitors (n=4–6).
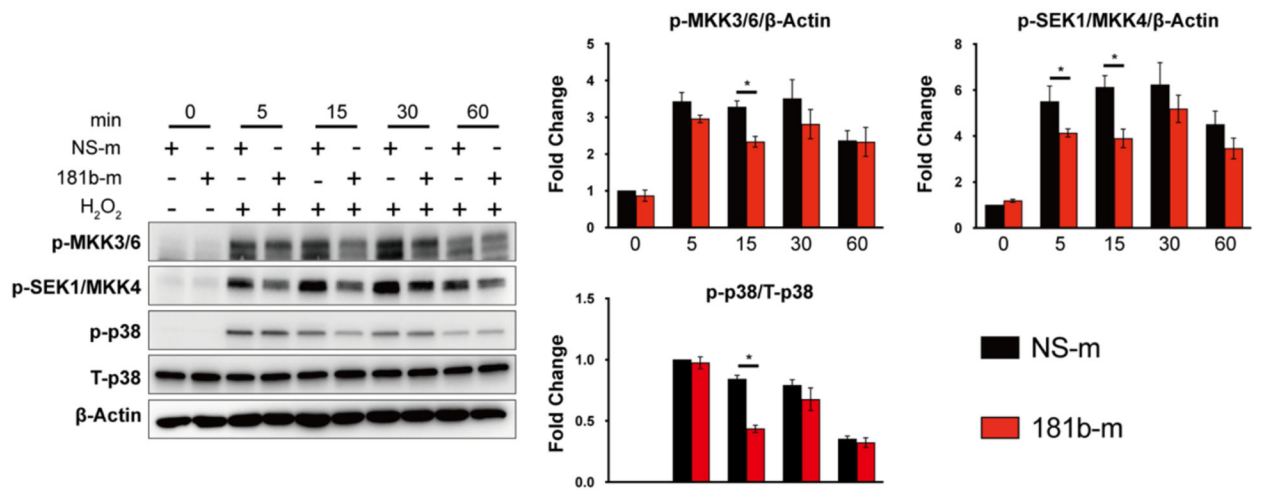
**Fig. 4. miR-181b regulates MKKs/MAPK signaling pathway**
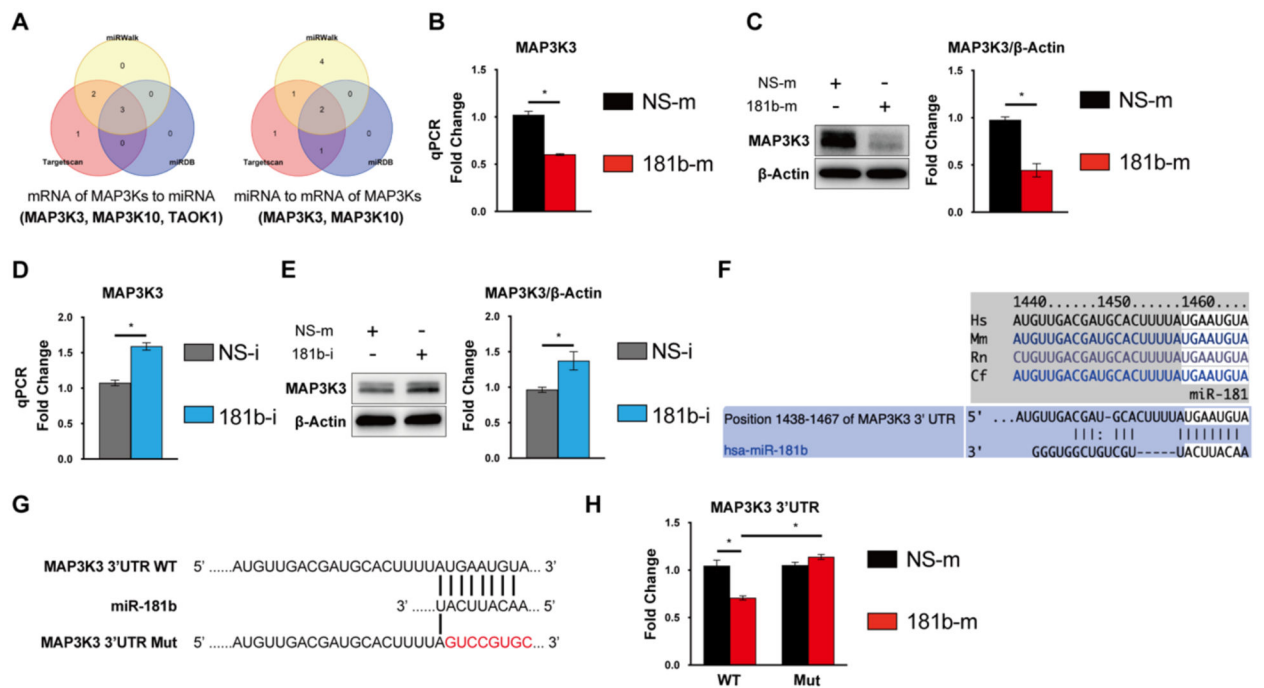**(A)** miR-181b overexpression reduces the phosphorylation of MKK3/6, SEK1/MKK, p38
(n=3).

**Fig. 5. miR-181b targets MAP3K3.**
**(A)** The genes of MAPK signaling pathway targeted by miR-181b were predicted by algorithms miRWalk, TargetScan, or miRDB. miR-181b mimics reduce mRNA expression (n=3) **(B)** and protein expression (n=3). **(C)** of MAP3K3. miR-181b inhibition increases mRNA expression (n=3). **(D)** and protein expression **(E)** of MAP3K3. **(F)** The binding site in MAP3K3 3'UTR for miR-181b was conserved across species and predicted by TargetScan3.1. **(G)** Schematic diagram of luciferase-reporter constructs containing wild-type or mutated 3'UTR of MAP3K3. **(H)** miR-181b inhibits MAP3K3 with WT 3'-UTR but not the MAP3K3 with the 3'-UTR mutation (n=4).
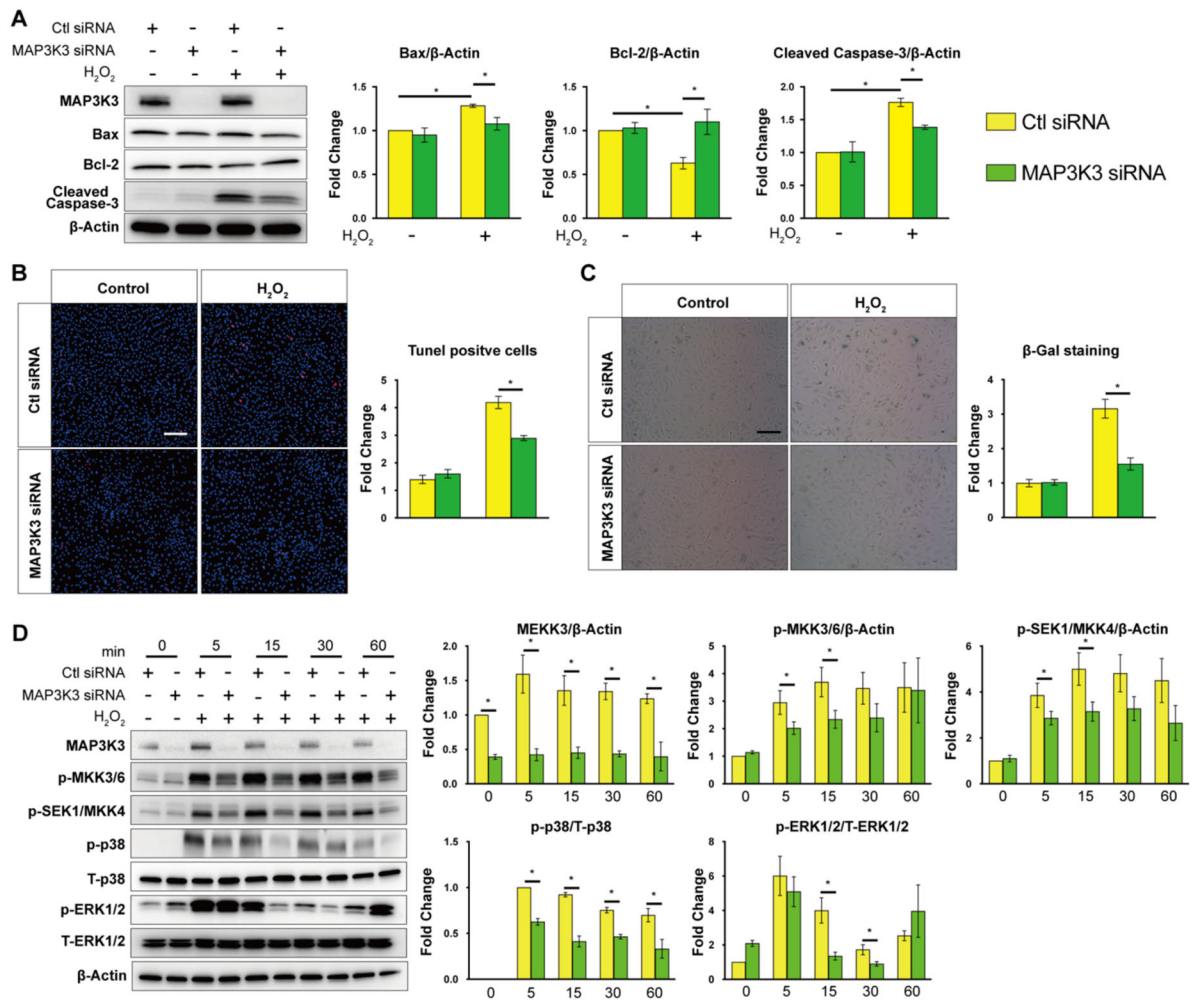
**Fig. 6. MAP3K3 regulates endothelial apoptosis and senescence via a MAPK signaling pathway.**
**(A)** MAP3K3 knockdown regulates protein expression of Bax, Bcl-2, cleaved-Caspase3 in ECs (n=3). **(B)** Quantification of TUNEL staining in siRNA-transfected HUVECs stimulated with $H_2O_2$ (1hr, 30μM) (n=4–5). **(C)** Quantification of SA-βgal staining in siRNA-transfected HUVECs stimulated with $H_2O_2$ (1hr, 30μM) (n=4–6). **(D)** MAP3K3 knockdown modulates the phosphorylation of MKK3/6, SEK1/MKK, p38, and ERK1/2 in ECs (n=3).
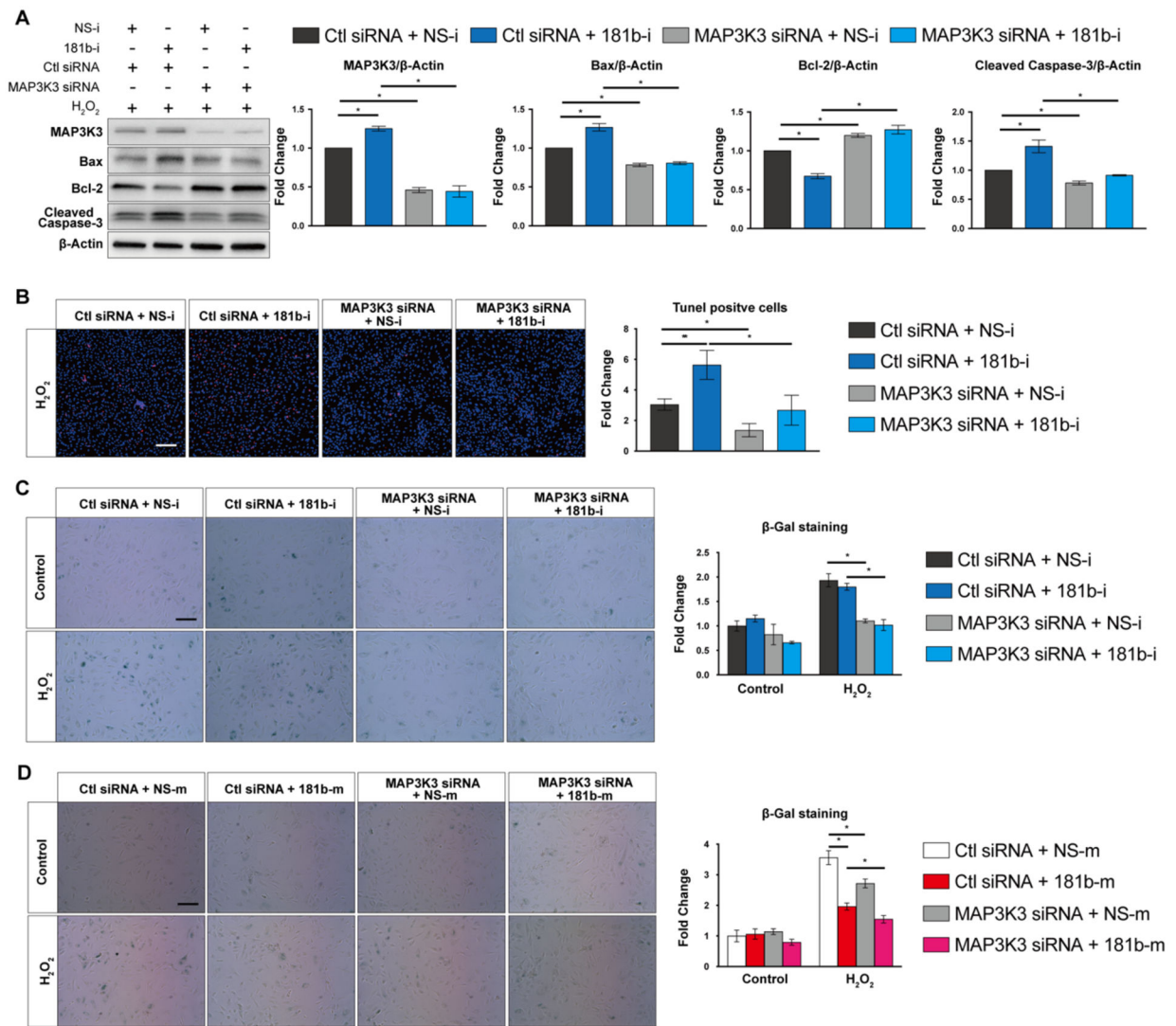
**Fig. 7. miR-181b is dependent on MAP3K3 for regulating EC apoptosis and senescence.**
**(A)** Dependency study of miR-181b inhibitor and MAP3K3 siRNA on protein expression of Bax, Bcl-2, and cleaved-Caspase-3 (n=3). **(B)** Effect of miR-181b inhibitor in the presence of MAP3K3 knockdown by siRNA on TUNEL staining in ECs (n=4–5). Effect of miR-181b inhibitor (n=4–6) **(C)** or mimic (n=4–6) **(D)** in the presence of MAP3K3 knockdown on SA-β-gal staining in ECs.
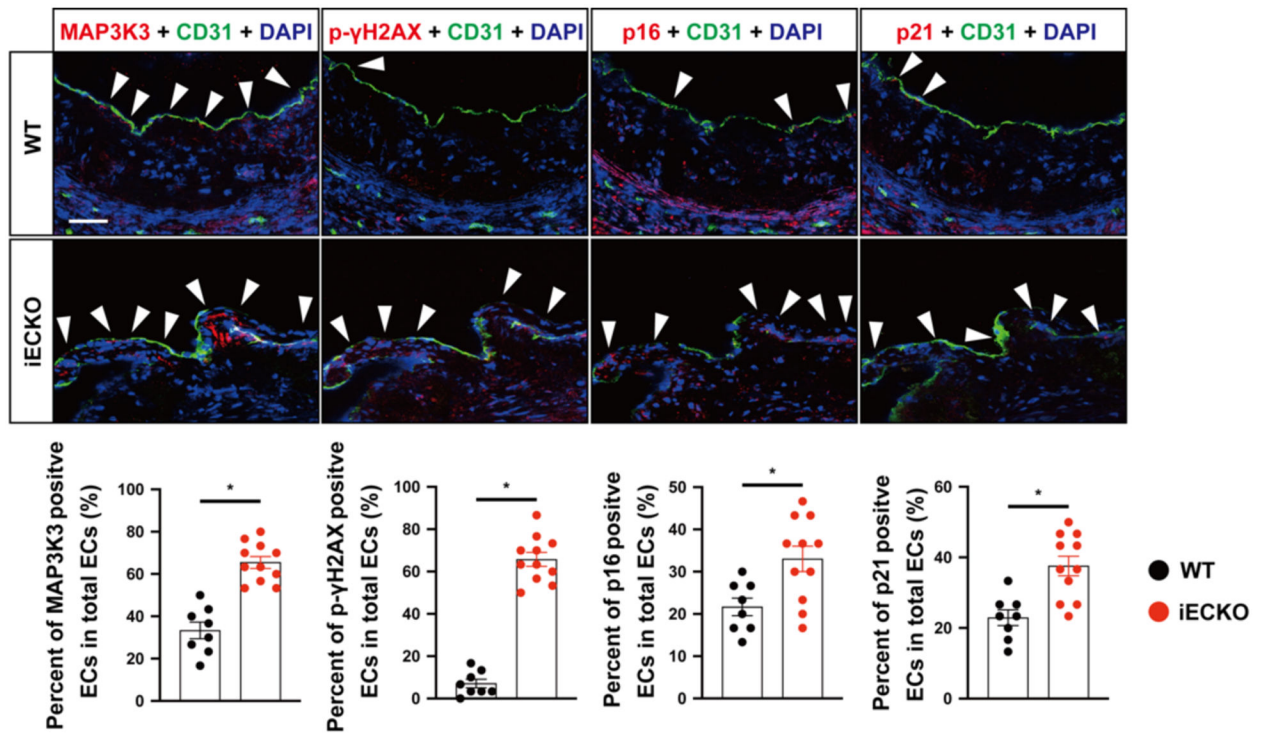
**Fig. 8. Endothelial-specific deficiency of miR-181a2b2 increases senescence markers in the aorta.** Immunofluorescence staining of p21, p16, γ-H2AX, MAP3K3 in the aorta sinus of miR-181a2b2 iEC KO old (n=11) or control mice (18 months) (n=8): representative images are shown on the left, statistical diagrams are shown on the right.