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SIRT6-mediated transcriptional suppression of MALAT1 is a key mechanism for endothelial to mesenchymal transition

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ABSTRACT

Background: Vascular aging has profound effects on cardiovascular diseases. Endothelial to mesenchymal transition (EndMT) is defined as the acquisition of mesenchymal characteristics by endothelial cells (ECs) and has been found induced in a model of ECs aging. However, whether EndMT occurs during aging *in vivo*, the functional significance of EndMT on vascular biology and the underlying mechanisms remain unknown.
Methods and results: In this study, we examined the vascular ECs from young (2 months old) and old (18 months old) mice, and demonstrated that aged ECs underwent EndMT. Moreover, the transwell assay showed that EndMT process was accompanied by increased endothelial permeability. It was found that sirtuin 6 (SIRT6), a nicotinamide adenine dinucleotide$^+$ (NAD^+)-dependent histone deacetylase, was down-regulated during ECs aging. Knockdown of SIRT6 in young ECs could induce EndMT. Next, we identified five long non-coding RNAs that are enriched in ECs for downstream effector of SIRT6; only metastasis associated lung adenocarcinoma transcript 1 (MALAT1) was significantly up-regulated in aged ECs. Knockdown of SIRT6 could increase MALAT1 levels. Furthermore, the ChIP assay and luciferase reporter gene assay confirmed that SIRT6 bound directly to the promoter region of MALAT1 and suppressed MALAT1 expression. Finally, we demonstrated that MALAT1 mediated aging-induced EndMT through increasing Snail expression.
Conclusion: Our study provides *in vivo* evidence that ECs undergo EndMT during vascular aging, which increases endothelial permeability. SIRT6-mediated transcriptional suppression of MALAT1 is a key mechanism for EndMT. Manipulating EndMT may be considered as a new therapeutic strategy for retarding aging-associated vascular diseases.

1. Introduction

The World Health Organization forecasts that the number of people aged 60 and older will reach two billion or more by the year 2050. Cardiovascular diseases (CVDs) are major aging-related diseases and mostly due to the vascular dysfunction. It would be urgently required to consider the control of vascular aging as our population ages.

Endothelial cells (ECs) play key roles in the regulation of vascular functions. EndMT is characterized by the loss of cell-cell adhesion and the generation of elongated, spindle-shaped mesenchymal cells, which was initially found during embryonic heart development [1] and contributes to various pathogenesis like tissue fibrosis [2], cancer progression [3], hypertension [4], atherosclerosis [5], and cerebral cavernous malformations [6]. Recently, Fleenor et al. found that the *in vitro* cultured human aortic ECs (HAECs) changed to mesenchymal cells during replicative culturing [7]. However, the *in vivo* correlation between EndMT and aging has not been identified. The mechanisms responsible for aging-induced EndMT are not investigated.

Sirtuin 6 (SIRT6) is a nicotinamide adenine dinucleotide$^+$ (NAD^+)-dependent histone deacetylase and is a key regulator of aging [8]. Studies have demonstrated that SIRT6 regulates endothelial functions like DNA damage and telomere dysfunction [9]. Based on these reports, we speculate that SIRT6 may play a role in the mediation of the aging-induced EndMT.

Recently, long non-coding RNAs (lncRNAs) has altered our understanding of the regulation of diseases and multiple biological

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processes, including differentiation, development, and metabolism [10–12]. In the field of aging, the discover of critical lncRNAs, such as H19, a regulator of skeletal muscle differentiation which is selectively up-regulated in old muscle [13] and BCYRN1, a biomarker of neurodegeneration which is decreased in brain of old individuals [14], has brought these heretofore neglected molecular players to the forefront of aging research. However, it is unknown whether certain lncRNAs are involved in the endothelial aging and the related EndMT process.

The goals of this study were as follows: 1) to investigate whether aged ECs undergo EndMT *in vivo*; 2) to investigate the roles of SIRT6 in aging-induced EndMT; 3) to identify lncRNAs as downstream targets of SIRT6 and its possible involvement in the observed aging-induced EndMT.

2. Material and methods

2.1. Mice

The healthy male C57BL/6 mice used in this study were housed at constant temperature of 23 ± 1 °C and humidity of 55 ± 5%. The mice were distributed into two groups: young (2 months old) and old (18 months old). Animals were sacrificed under terminal anaesthetic (isoflurane >3% in 95% O_2 and 5% CO_2). All of the experimental procedures were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by The Harbin Medical University Animal Care and Use Committee.

2.2. Cell culture and transfection

HAECs (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in ECs medium (ECM) supplemented with 5% fetal bovine serum, 1% ECs growth factors, and 1% penicillin/streptomycin. Population doubling level (PDL) was calculated to determine the age of the HAECs. The following formula was used for the calculation of PDL: $n = \log_2 (C_H/C_S) + X$, in which n = the final PDL number at the end of a given subculture, C_H = the number of viable cells at harvest, C_S = the number of cells seeded, and X = the doubling level when the subculture begins. Cell morphology was observed using an inverted phase contrast microscope and images were acquired using a camera. The knockdown of MALAT1 and SIRT6 expression was performed using small-interfering RNA (siRNA) synthesized by Invitrogen (Carlsbad, CA, USA).

2.3. Analysis of vascular senescence, histology and immunohistochemistry, intimal RNA isolation from aorta, senescence-associated β-galactosidase (SA-β-gal) staining, western blot, real-time reverse transcription-polymerase chain reaction (real-time RT-PCR), immunofluorescence, measurement of endothelial permeability, chromatin immunoprecipitation (ChIP) assay, luciferase reporter activity assay

The detailed processes of these experiments are shown in Supplementary Methods.

2.4. Data analysis

All data in this study are expressed as mean ± SD. Statistical comparisons among two groups were performed by two-tailed student's *t*-test. For multiple groups, one-away ANOVA accompanied with Turkey multiple-comparisons test was used (GraphPad Prism version 6.0). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. EndMT occurred in aged ECs in vitro

We established an endothelial replicative senescence model by continuous passage culture of HAECs. Senescent status was verified by *in situ* senescence-associated β-gal staining. β-gal-positive cells were markedly increased in old HAECs (PDL 32) *versus* young cells (PDL 16) (Supplementary Fig. 1A). Old HAECs exhibited characteristic signs of aging, including a flat and enlarged morphology. Notably, we also found an obvious cell shape change from a round, cobblestone-like phenotype to an elongated, spindle-shaped phenotype (Supplementary Fig. 1A). Western blot analysis was performed to assess the protein expression of endothelial markers CD31 and VE-cadherin, and mesenchymal markers α-SMA and FSP1 in young and old HAECs. The protein levels of p53 and p21 were measured to indicate cellular senescence. We found that CD31 and VE-cadherin were significantly down-regulated, while p53, p21, α-SMA and FSP1 were up-regulated in old HAECs, illustrating the acquisition of the mesenchymal phenotype during ECs aging (Supplementary Fig. 1B). We further confirmed the involvement of EndMT by immunofluorescence labeling and real-time RT-PCR (Supplementary Fig. 1C and D). Moreover, results from transwell assays demonstrated that the permeability of ECs increased significantly during aging (Supplementary Fig. 1E and F).

3.2. EndMT occurred in aged vascular endothelium in mice

To study the possible changes of vascular endothelium in aging body, young and old C57BL/6 mice were used. Mice designated as "young" were 2 months of age and mice designated as "old" were 18 months of age [15]. There was an obvious age-dependent weight gain in mice (Fig. 1A). We performed *en face* whole aorta analysis of vascular aging using β-gal staining [16]. An increase in β-gal stained area was observed in the aorta of the aged mice compared to their younger counterparts (Fig. 1B). Fig. 1C showed the analysis of histological sections of the aortic sinus stained with hematoxylin and eosin (HE). To examine whether EndMT occurs during vascular aging, we performed immunofluorescence double-labeling experiments in the aortic sinus of mice using antibodies to the endothelial marker CD31 and the mesenchymal marker α-SMA. Cells expressing both CD31 and α-SMA were obvious in aged mice, but such double-positive cells were rarely detected in their younger counterparts (Fig. 1D). The summarized data of fluorescence signals in endothelial cells was shown in Fig. 1E. To gather further evidence for the acquisition of a mesenchymal phenotype of ECs, aortic intima was harvested for PCR analysis. CD31 was robustly enriched, and smooth muscle myosin heavy chain (smMHC) was much lower in the aortic intima compared to the media plus adventitia, which indicates that the isolated aortic intima contained highly pure ECs (Fig. 1F). We found that the endothelial markers CD31 and VE-cadherin were decreased and the mesenchymal markers α-SMA and smMHC were increased in the endothelium of aged mice compared to young mice (Fig. 1G). Taken together, these data demonstrated that EndMT occurred during vascular aging in mice.

3.3. SIRT6 mediated aging-induced EndMT

SIRT6 has been reported to be a negative regulator of cellular senescence [17]. We first measured the expression of SIRT6 in ECs. Using immunostaining of sections from the aortic sinus, we found that SIRT6 was decreased in the endothelium of old mice (Fig. 2A). PCR analysis of aortic intima RNA also demonstrated significant down-regulation of SIRT6 in old mice (Fig. 2B). We also measured SIRT6 protein and mRNA levels in young and old HAECs. Notably, SIRT6 expression was lower in old HAECs (Fig. 2C and D). To further determine the role of SIRT6 in EndMT, we silenced SIRT6 expression by siRNA in young HAECs, with confirmation of mRNA expression by PCR (Fig. 2E) and protein

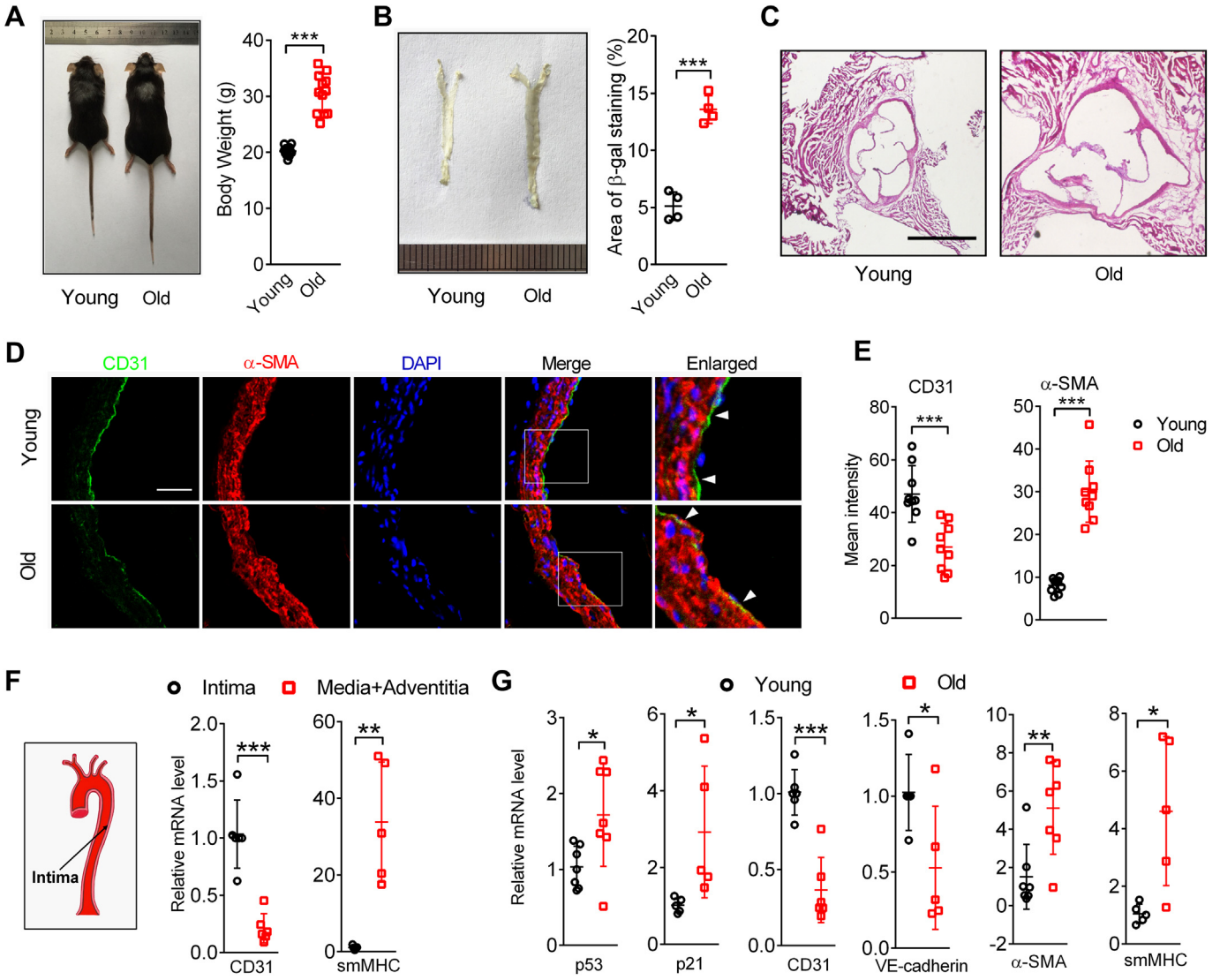

Fig. 1. EndMT occurred in aged vascular endothelium in mice. (A) Pictures and body weight of young (2 months) and old (18 months) C57BL/6 mice. $n = 12$. (B) Representative aorta *en face* macrographs of senescence (β-gal staining) and the percentage of senescence area. $n = 4$. (C) HE staining of aortic root sections. Scale bar: 300 μm. (D) Confocal immunofluorescence double-labeling in aortic roots of young and old mice with antibodies to CD31 (green) and α-SMA (red). The nuclei were stained in blue with DAPI. White arrowheads denote colocalization of CD31 and α-SMA expression in the aortic intima. Scale bar: 50 μm. (E) The CD31 and α-SMA expressions in ECs were quantified to reflect the EndMT process. $n = 9$. (F) Diagrammatic drawing of the aorta. ECs marker CD31 and smooth muscle cell marker smMHC expressions were detected using real-time RT-PCR in the aortic intima, media and adventitia. $n = 5$–6. (G) mRNA levels of senescence markers (p53 and p21), ECs markers (CD31 and VE-cadherin), and mesenchymal cell markers (α-SMA and smMHC) using real-time RT-PCR in the aortic intima. $n = 5$–7. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression by western blot (Fig. 2G). Knockdown of SIRT6 altered the cell morphology from a cobblestone-like endothelial phenotype to a spindle-shaped mesenchymal phenotype (Fig. 2F). Western blot and PCR analysis compared EndMT-related gene expression in cells with or without knockdown of SIRT6. We found that endothelial markers CD31 and VE-cadherin were significantly down-regulated, while the mesenchymal markers α-SMA and FSP1 were significantly up-regulated in cells with knockdown of SIRT6 (Fig. 2G and I). Immunofluorescence staining confirmed that cells with SIRT6 knockdown underwent EndMT (Fig. 2H). The silencing of SIRT6 increased ECs permeability, as suggested by the transwell permeability assay (Fig. 2J). These results demonstrated that the down-regulation of SIRT6 was critical for aging-induced EndMT.

3.4. SIRT6 negatively regulated metastasis associated lung adenocarcinoma transcript 1 (MALAT1) expression

Emerging evidence indicates that lncRNAs are key regulators of endothelial biology [18]. To investigate the possible involvement of lncRNAs in the observed aging-induced EndMT, we focused on 5 conserved lncRNAs that are highly expressed in ECs, namely linc00493, linc00657, maternally expressed 3 (MEG3), taurine upregulated gene 1 (TUG1) and MALAT1 [19]. PCR analysis indicated that MALAT1 was profoundly up-regulated during HAEC aging, but the expressions of the other 4 lncRNAs were not significantly altered (Fig. 3A). The up-regulation of MALAT1 during aging was further confirmed *in vivo* by examining MALAT1 expression in aortic intima from aged mice relative to the young counterparts (Fig. 3B). Knockdown of SIRT6 in HAECs enhanced MALAT1 expression (Fig. 3C). We experimentally verified whether SIRT6 binded to the promoter regions of MALAT1 using ChIP assay. SIRT6 immunoprecipitates were highly enriched in the promoter of MALAT1 fragment and silencing of SIRT6 demonstrated a significant absence of the expected band (Fig. 3D). To further explore the regulatory effect of SIRT6 on MALAT1, we subcloned the promoter fragment of MALAT1 into the pGL3 basic firefly luciferase reporter plasmids and transfected the plasmids with SIRT6-specific siRNA into HAECs. The inhibition of SIRT6 dramatically increased the luciferase activity, while transfection of control siRNA had no effect (Fig. 3E). These results

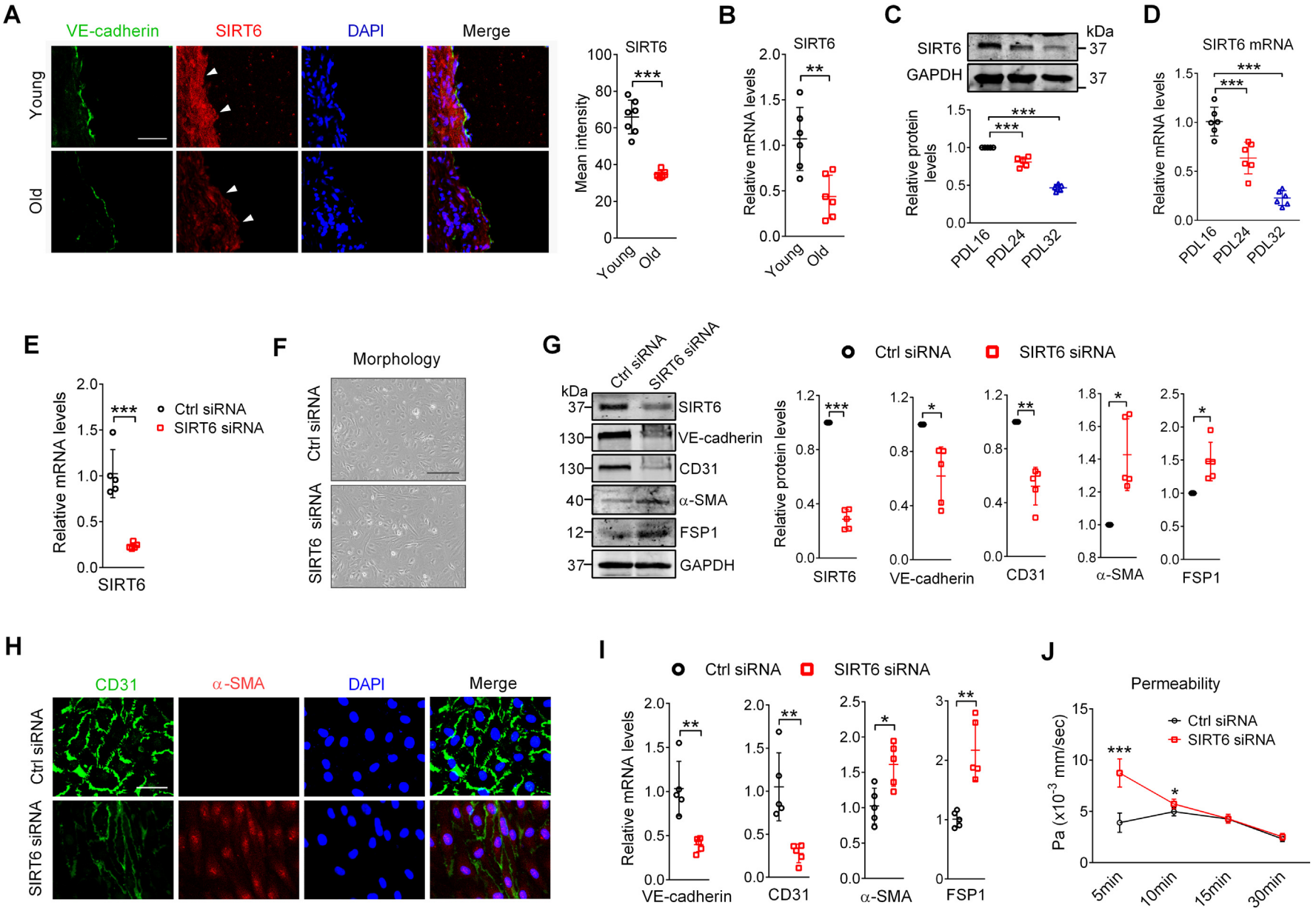

Fig. 2. SIRT6 decreased with aging and mediated aging-induced EndMT. (A) Frozen sections of aortic root were stained for SIRT6 (red) and VE-cadherin (green). Arrowheads indicated differential SIRT6 expression in ECs. The nuclei were stained blue with DAPI. Scale bar: 50 μm. SIRT6 expression in vascular ECs was quantified. $n = 7$ mice each group. (B) SIRT6 mRNA levels in aortic intima were measured using real-time RT-PCR. $n = 6$ mice each group. (C) Protein levels of SIRT6 in young and old HAECs. $n = 5$. (D) mRNA levels of SIRT6 in young and old HAECs. $n = 6$. (E) mRNA levels of SIRT6 in young HAECs after transfection with control siRNA or SIRT6 siRNA. $n = 5$. (F) Cell morphology changes after transfection with control siRNA or SIRT6 siRNA in HAECs. Scale bar: 300 μm. (G) Western blot analysis of SIRT6, VE-cadherin, CD31, α-SMA and FSP1 after transfection with control siRNA or SIRT6 siRNA in HAECs. $n = 5$. (H) CD31 and α-SMA expression was detected by immunofluorescence in HAECs transfected with control siRNA or SIRT6 siRNA. Scale bar: 50 μm. (I) mRNA levels of SIRT6, VE-cadherin, CD31, α-SMA and FSP1 in HAECs after transfection with control siRNA or SIRT6 siRNA. $n = 5$. (J) Permeability coefficients of HAECs were examined using a transwell permeability assay. Pa indicates permeability coefficient of albumin. $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

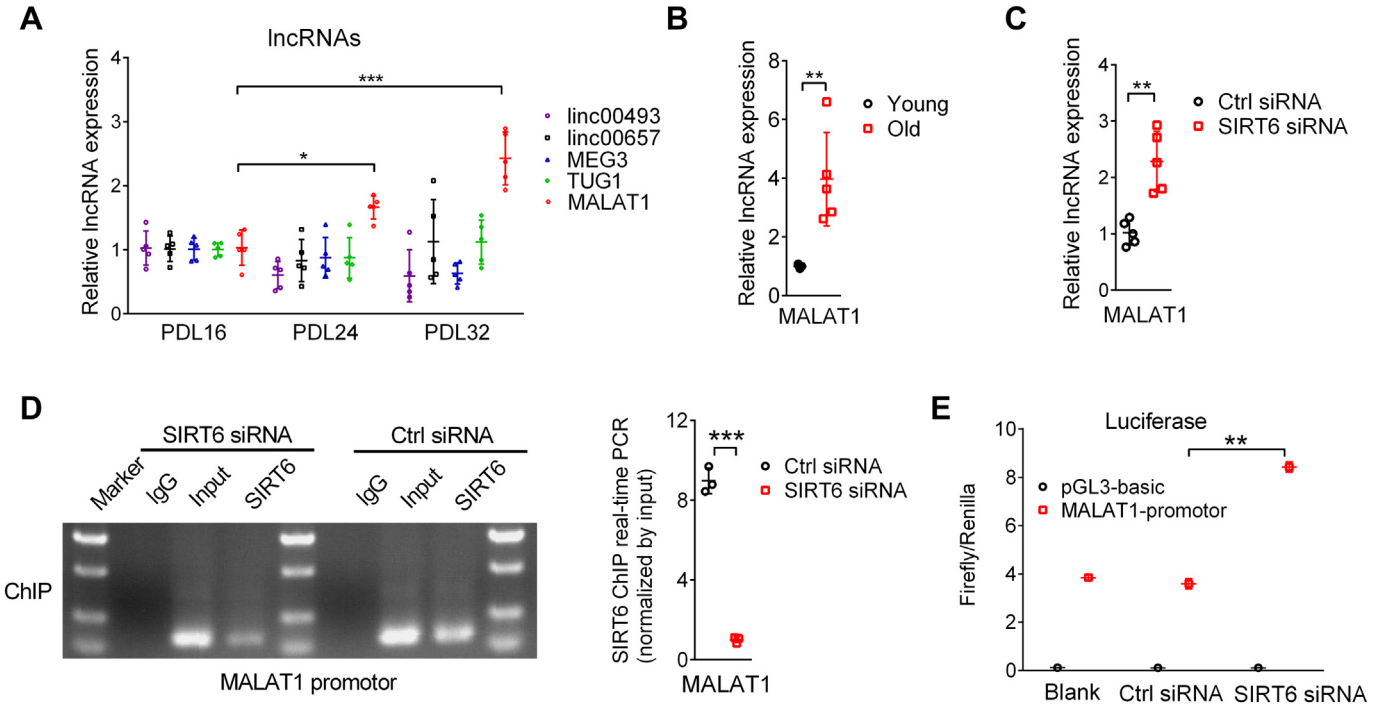

Fig. 3. SIRT6 negatively regulated MALAT1 expression. (A) lncRNAs expressions in young and old HAECs were measured using real-time RT-PCR. $n = 5$. (B) Real-time RT-PCR analysis of MALAT1 levels in the aortic intima of young and old mice. $n = 5$. (C) Effect of SIRT6 knockdown on MALAT1 expression in HAECs. $n = 5$. (D) SIRT6 binding at the promoter region of MALAT1 and the effect of SIRT6 depletion on this binding were assessed using ChIP asssay. ChIP-derived DNA was amplified by real-time RT-PCR using specific primers for MALAT1 promotor. $n = 3$. (E) Effect of SIRT6 depletion on MALAT1 promoter activity was evaluated using a luciferase reporter assay. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

suggested that SIRT6 binded directly to the MALAT1 promoter and negatively regulated MALAT1 expression in HAECs.

3.5. MALAT1 modulated aging-induced EndMT via snail

We designed experiments to address the role of MALAT1 in aging-induced EndMT. MALAT1 was knocked down in old HAECs (PDL 30) using siRNA, with significant reduction in MALAT1 expression (Fig. 4A). HAECs with knockdown of MALAT1 displayed an endothelial cobblestone appearance compared to control cells, which exhibited an elongated mesenchymal morphology (Fig. 4B). Moreover, we found that the protein and mRNA expressions of endothelial markers CD31 and VE-cadherin were markedly increased, but the mesenchymal markers α-SMA and FSP1 were significantly reduced in MALAT1-silenced cells (Fig. 4C and E). The results from immunofluorescence staining revealed that the expression of CD31 was significantly increased and α-SMA was markedly decreased in MALAT1-silenced cells (Fig. 4D). The transwell permeability assay demonstrated that the permeability coefficient of MALAT1-silenced cells was lower than that in the control ones (Fig. 4F). To gain insight into the mechanisms by which MALAT1 regulates EndMT, we examined the expression of Snail, a key transcriptional regulator of EndMT [20]. Western blot and PCR analysis demonstrated that MALAT1 knockdown decreased Snail expression in old HAECs (Fig. 4G and H). These data showed that MALAT1 mediated aging-induced EndMT *via* the up-regulation of Snail.

4. Discussion

Vascular aging has profound effects on cardiovascular disease [21]. ECs are one of the main cell types that compose blood vessels. Several studies have found that ECs aging is associated with increased monolayer permeability and endothelial barrier dysfunction [22,23]. However, the reasons for these observations are not clear. EndMT weakens the intercellular adhesive force and enhances the intracellular contraction force that contributes to aging-induced endothelial hyperpermeability, leading to increased infiltration of inflammatory cells in cardiovascular events and can also contribute to deteriorated tumor microenvironment [24]. Therefore, our findings that EndMT occurs during aging may partially explain the increased susceptibility for cardiovascular diseases and cancer in the elderly.

SIRT6 is a crucial player in aging [25,26]. Interestingly, a recent study found that SIRT6 is not the same in every species. By analyzing DNA repair in 18 rodent species with lifespans ranging from 3 years (mice) to 32 years (beavers), Tian et al. found that the rodents with longer lifespans have more efficient SIRT6 proteins, resulting in more efficient DNA repair ability. They identified five amino acids (residues 235, 249, 260, 263, and 264) responsible for making the stronger SIRT6 protein in beavers compared with mice. When all the available SIRT6 sequences are aligned, they found the long-lived species tend to have Histidine in position 249 and Threonine in position 263 while short-lived species have Glutamine in position 249 and Cysteine or Serine in position 263 [27]. We found that within the amino acids 220–270 region, human SIRT6 protein has Histidine in position 249 while mouse has Glutamine. Human SIRT6 protein has Threonine in position 263 while mouse has Cysteine (Supplementary Fig. 2), which may be a reason for the longer lifespan of human.

An important finding in our study is that SIRT6 is a key inducer for EndMT. We found that SIRT6 expression (mRNA and protein) was significantly down-regulated in aged ECs both *in vivo* and *in vitro*, confirming a link between SIRT6 and endothelial aging. As SIRT6 has been shown to be involved in regulating endothelial dysfunction, including eNOS function, DNA damage, and telomere dysfunction [9,28], we hypothesized that SIRT6 had a key role in mediating aging-induced EndMT. The results of this study demonstrated that knockdown of SIRT6 in young ECs induced a mesenchymal phenotype, supporting our hypothesis.

Our findings that SIRT6 is a key inducer for EndMT stimulated our interest in exploiting the downstream effector of SIRT6. We examined five lncRNAs that are highly expressed in ECs and found that only MALAT1 expression was significantly increased during aging. We hypothesized that MALAT1 was a downstream effector of SIRT6. SIRT6 is a

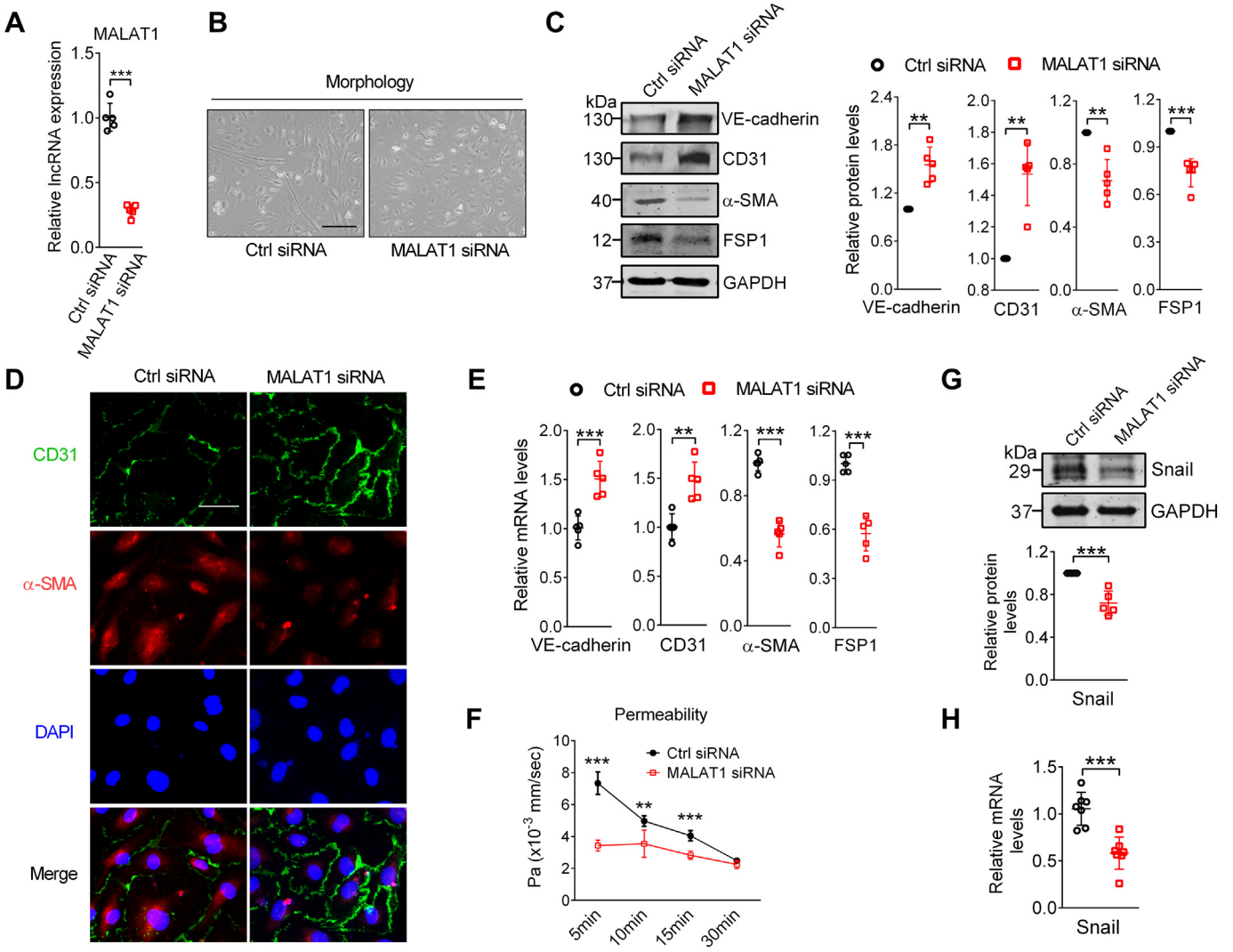

Fig. 4. MALAT1 mediated aging-induced EndMT *via* Snail. (A) MALAT1 levels were measured after transfection with control siRNA or MALAT1 siRNA in old HAECs. $n = 5$. (B) Cell morphology changes after depletion of MALAT1 in old HAECs. Scale bar: 300 μm. (C) Western blot analysis of VE-cadherin, CD31, α-SMA and FSP1 after depletion of MALAT1 in old HAECs. $n = 5$. (D) CD31 and α-SMA expressions were detected using immunofluorescence in old HAECs after depletion of MALAT1. Scale bar: 50 μm. (E) Real-time RT-PCR analysis of VE-cadherin, CD31, α-SMA and FSP1 levels in old HAECs after depletion of MALAT1. $n = 5$. (F) Permeability coefficients of HAECs were examined using a transwell permeability assay. Pa indicates permeability coefficient of albumin. $n = 5$. (G) Western blot analysis of Snail after depletion of MALAT1. $n = 5$. (H) Real-time RT-PCR analysis of Snail in old HAECs after depletion of MALAT1. $n = 7$. $^{**}p < 0.01$, $^{***}p < 0.001$.

chromatin-associated protein that is known for its NAD^+-dependent deacetylation activity, which suppresses the expression of target genes [29]. This study demonstrated that SIRT6 directly bound to the promoter region of MALAT1 and repressed MALAT1 expression in ECs. These actions explain why MALAT1 expression increases during endothelial aging.

MALAT1 has been proved to play a role in EndMT. Xiang et al. demonstrated that MALAT1 modulates TGF-β1-induced EndMT through regulation of TGFBR2 and SMAD3 *via* miR-145 [30]. Others found MALAT1 mediates ox-LDL induced EndMT through the Wnt/β-catenin signaling [31]. However, whether MALAT1 has a role in aging-induced EndMT is unknown. The results of this study demonstrated that knockdown of MALAT1 dramatically attenuated EndMT in aged ECs. We also found MALAT1 knockdown attenuated Snail expression, an EndMT master regulatory transcription factor. These results indicate that MALAT1 modulates aging-induced EndMT at least partially through Snail.

4.1. Study limitations

This study had a major limitation that needs to be addressed. Although the SIRT6/MALAT1 pathway was shown to be involved in aging-induced EndMT *in vitro*, the clinical study regarding the involvement of the SIRT6/MALAT1 pathway in EndMT is not verified. These issues will be solved using proper animal models in our future studies.

4.2. Conclusions

In conclusion, our study uncovers EndMT as a novel cell biology mechanism for the endothelial senescence and SIRT6/MALAT1 as a novel epigenetic mechanism for regulation of EndMT. Manipulating EndMT through modulating SIRT6 and MALAT1 can be considered as a new therapeutic strategy for retarding aging associated vascular diseases.

Acknowledgments

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Conflict of interest

The authors have declared that no competing interest exist in this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.07.082.

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