Original Research Article

MicroRNA miR-214-5p induces senescence of microvascular endothelial cells by targeting the JAG1/Notch signaling pathway

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ABSTRACT

During cellular senescence, irreversible cell cycle arrest is accompanied by morphological and genetic alterations. MicroRNAs (miRNAs) play a critical role in regulating senescence by modulating the abundance of crucial senescence regulatory proteins. Therefore, to identify novel senescence-associated miRNAs, we analyzed differentially expressed miRNAs in microvascular endothelial cells (MVEC). Among the 80 differentially expressed miRNAs in replicative senescent MVECs, 16 miRNAs of unknown gene ontology were used in the senescence-associated β-galactosidase assay. Thus, we identified miR-214-5p as having high senescence-inducing activity, inhibiting the proliferation and angiogenesis activity of MVECs. To reveal the senescence-regulating mechanism of miR-214-5p, we searched for target genes through sequence- and literature-based analysis. Molecular manipulation of miR-214-5p demonstrated that miR-214-5p regulated the expression and function of Jagged 1 (JAG1) in senescent MVECs. Silencing *JAG1* or downstream genes of JAG1-Notch signaling, accelerated the senescence of MVECs. Additionally, ectopic overexpression of JAG1 reversed the senescence-inducing activity of miR-214-5p. In conclusion, we identified miR-214-5p as a senescence-associated miRNA. Targeting miR-214-5p may be a potential strategy to delay vascular aging and overcome the detrimental effects of senescence and age-related diseases.

1. Introduction

Vascular endothelial cells are arranged as single-cell layers constituting the vascular lumen that play an essential role in maintaining vascular homeostasis, such as angiogenesis, blood coagulation, and inflammatory immune response [1]. Vascular endothelial cells can undergo senescence and dysfunction owing to various environmental and genetic risk factors [2]. Dysfunction of endothelial cells can contribute to cardiovascular diseases (CVD), such as atherosclerosis, hypertension, stroke, and coronary artery disease. The incidence of CVD has recently increased with the aging population. As endothelial senescence is a risk factor for CVD [3], we investigated ways to prevent and control CVD by regulating the senescence of vascular endothelial cells.

Hayflick and Moorhead reported in 1961 that normal human cells possess a limited capacity for approximately 60 cell divisions [4]. When this limit is exceeded, cells enter irreversible growth arrest, known as replicative senescence. In senescent cells, various cellular and molecular changes occur [5], which are characterized by a flat and enlarged cellular morphology [6]. Due to the increase in intracellular reactive oxygen species (ROS) levels and DNA damage, cell growth is arrested and no longer divides [7–9]. Moreover, senescent cells secrete various growth factors, cytokines, and proteinases in the surrounding area, referred to as the senescence-associated secretory phenotype (SASP) [10]. In addition to the aforementioned physiological alterations, changes in gene expression profiles occur, including small RNA expression [11].

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNA with an average length of approximately 22 nucleotides. MiRNAs function through base pairing with complementary sequences within mRNA molecules, resulting in the post-transcriptional regulation of gene expression [12]. MiRNAs are associated with various biological functions such as development, cell proliferation, cell fate determination, and regulation of cellular mechanisms [13,14]. Furthermore, numerous miRNAs influence senescence by modulating the abundance of crucial senescence regulatory proteins [15–17]; these are known as senescence-associated miRNAs (SA-miRNAs) [17–19]. Therefore, understanding the molecular mechanisms of SA-miRNAs is vital to developing diagnostic tools and therapeutic opportunities for various diseases.

The Notch signaling pathway mediates the interaction between adjacent endothelial cells (ECs) to regulate differentiation, embryonic development, and proliferation [20]. Mammalian cells express four transmembrane Notch receptors (Notch 1–4) and five transmembrane ligands [Jagged (JAG) 1, 2 and Delta-like canonical notch ligand (DLL) 1, 3, and 4], that are abundantly expressed in ECs [20,21]. The Notch ligands JAG1 and DLL4 are antagonistic. They are involved in angiogenesis and the development of vasculature through their balance [22]. In addition, endothelial-specific deletion of JAG1 causes embryonic lethality and cardiovascular defects [21]. Moreover, overexpression of JAG1 in tumors can promote angiogenesis [23]. Regarding cellular senescence, it has been reported that JAG1 expression decreases with aging in ECs [20], and inhibition of Notch signaling induces premature senescence via a p16-dependent pathway [20,24]. However, the mechanism of JAG1 downregulation during cellular senescence has not yet been fully elucidated.

Previously, we reported that SA-miRNAs downregulate the high mobility group protein B2 (HMGB2) and contribute to cellular senescence in the microvascular endothelial cells (MVECs) [25]. Here, we focused on identifying more SA-miRNAs in MVECs as potential diagnostic means and promising therapeutic targets to delay vascular aging.

2. Material and methods

2.1. Cell culture

Human lung MVECs were purchased from Cell Applications (San Diego, CA, USA) and cultured in EBM-2 medium supplemented with EGM-2MV (Lonza, Hopkinton, MA, USA). In total, 5×10^5 cells were plated in a 100-mm culture plate and cultured at 37 °C in a humidified incubator with 5% CO_2. The cells were passaged every 3 days via trypsinization. Cell counts were determined at the end of every passage after staining with 0.1% trypan blue using a hemocytometer. The cumulative number of population doublings (PDLs) was calculated in relation to the initial cell number.

2.2. Screening of differentially expressed miRNAs in senescent MVECs

The small RNA sequencing data were retrieved from the GEO database (GSE192677) and bioinformatic data analysis were performed by ebiogen (Seoul, Korea) as previously described [25].

2.3. Transfection of small interfering RNA (siRNA), miRNA, and miRNA inhibitors

The siRNAs and synthetic miRNA mimics used in this study were synthesized by Genolution Pharmaceuticals (Seoul, Korea). miRNA inhibitors involving 2′-O-methyl-modified oligoribonucleotide single strands were purchased from Genolution Pharmaceuticals and Integrated DNA Technologies (Singapore). MVECs (1.5×10^5 cells) were reverse-transfected with 20 nM siRNA, miRNA, or 50 nM miRNA inhibitor using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). The sequences of the siRNAs and miRNA mimics are listed in Supplementary Table S1.

2.4. Senescence-associated β-galactosidase (SA-β-gal) staining and SPiDER-β-gal assay

Cellular SA-β-gal activity was measured as previously described [8]. After taking at least six images per well at random locations, the numbers of SA-β-gal positive cells were counted manually. Cellular senescence was confirmed by measuring β-galactosidase activity using a SPiDER β-gal kit (Dojindo, Kumamoto, Japan). Briefly, 5×10^5 cells were suspended in phosphate buffered saline (PBS) (with 1% fetal bovine serum), diluted 1:1000 with a 1 mM stock solution of SPiDER β-gal, and incubated for 15 min at 37 °C. The proportion of senescent cells with high β-galactosidase activity was analyzed using a flow cytometer.

2.5. Quantitative reverse transcription PCR (qRT-PCR)

Quantitative reverse transcription PCR was performed as previously described [25]. To analyze miRNA expression, cDNA was synthesized using the Mir-X miRNA First-Strand Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA). Quantitative PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems). The miRNA expression was normalized to that of the reference genes (U6 control). The primers used for mRNA and miRNA qRT-PCR are listed in Supplementary Table S2. All fold changes were calculated using the ΔΔCt method.

2.6. Tube formation assay

Tube formation activity was evaluated as previously described [26, 27]. Briefly, 5×10^4 MVECs were seeded onto 24-well plates pre-coated with BD Matrigel Matrix (BD Biosciences, San Jose, NJ, USA) and incubated overnight to allow the formation of tube-like structures. Time-lapse images of EC tube formation were acquired using an InCell Analyzer 2000 (GE Healthcare Life Sciences, Little Chalfont, UK) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with the Angiogenesis Analyzer plugin.

2.7. Luciferase reporter assay

The 3′-UTRs (Untranslated regions) of human JAG1 (NM_000214.3) were subcloned into the 3′ region of the luciferase gene of the pGL3UC luciferase reporter vector [28]. The primer sequences used for amplification of the JAG1 3′-UTR were 5′-AAACTCTCTAGACAGACCGCG GGCACTGC-3′ and 5′-GAGGCCACACTGTTCCATGT-3′. Mutations in JAG1 3′-UTR were introduced by site-directed mutagenesis using the primers 5′-GCTTTAGACTTGAAAAGAGACTCCGAGGTGATCTGCTGC AGGCT-3′ and 5′-AGCCTGCAGCAGATCACCTCGGAGTCTCTT TTCAAGTCTAAAGC-3′. The 293T cells were seeded in 24-well plates and co-transfected with reporter plasmid pGL3UC-JAG1 3′-UTR (200 ng), pRL-CMV-Renilla plasmid (2 ng), and miRNAs (20 nM) using Turbofect™ transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the Renilla luciferase activity.

2.8. Western blot analysis

Cells were lysed in RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and protease inhibitors] and briefly sonicated. Protein content was measured using the Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), and equal amounts of cell lysate were separated via SDS-polyacrylamide gel electrophoresis and

transferred to nitrocellulose membranes (GE Healthcare Biosciences, Foster City, CA, USA). Membranes were immunoblotted with antibodies against KLF5 (Novus, Littleton, CO, USA), Hairy/Enhancer-of-split related with YRPW motif protein 1 (HEY1; Abcam, Cambridge, UK), Hairy/Enhancer of Split-1 (HES1; Cell Signaling, Berkeley, CA, USA), and JAG1 and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), and detected via chemiluminescence using enhanced chemiluminescence (ECL) detection reagents.

2.9. Overexpression of JAG1

The JAG1 gene of human origin (BC126205) was purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The primer sequences for the amplification of JAG1 were 5′- GTGCTGCTCGAG-CACCATGCGTTCCCCACGGACGCG-3′ and 5′- AATGCCGCGGCCGCC-TATACGATGTACTCCATTC-3′. The PCR-amplified JAG1 fragment was cut using XhoI and NotI restriction endonucleases and subcloned into the pLNCX2 vector (Clontech Laboratories, Inc., Mountain View, CA, USA). Retroviruses were generated in a 293GPG packaging cell line and transduced into MVECs in the presence of 4 μg/mL polybrene. After 2 d of incubation, transduced cells were aliquoted for western blotting, SA-β-gal staining, proliferation assay, and tube formation assay. Antibiotics selection was not applied, which could induce stress-induced senescence.

2.10. Statistical analysis

Data are presented as mean ± standard error. All results were analyzed for statistical significance using one-way and two-way analysis of variance (ANOVA) with Fisher's PLSD post-hoc test. P-values of 0.05 (*), 0.01 (**), and 0.001 (***) were considered as significant and were compared with those of the control group. Statistical analyses were performed using StatView, version 5.0.1.

3. Results

3.1. Screening of SA-miRNAs in MVECs

To identify unidentified SA-miRNAs, we analyzed small RNA sequencing data obtained from the replicative senescent MVECs model (GSE192677, [25]). The screening criteria for SA-miRNAs were as follows: 1) an increase of ≥1.3 times in old MVECs compared to that in young MVECs, and 2) raw data reading of $\log_2 \geq 4$. Eighty miRNAs whose expression increased simultaneously in two or more of the three

groups were selected (Fig. 1A, Supplementary Table S3). Among the selected miRNAs, those whose gene ontology (GO) was not known were selected (Fig. 1B). Through literature search, we excluded eight more miRNAs whose target genes were known to regulate cell growth or proliferation (Supplementary Table S4). Finally, 16 SA-miRNA candidates were further used in functional study. To investigate whether the selected miRNAs could induce premature senescence in young MVECs, we transfected a mimic of each miRNA into young cells and performed SA-β-gal assay (Fig. 1C). Compared with the negative control (miR-Cont, black), six miRNAs significantly induced senescence under these experimental conditions. We used miR-34a-5p (red), a well-known senescence-inducing miRNA [19,29], as a positive control. Among these, we selected miR-214-5p to further study its mechanism of action.

3.2. miR-214-5p is a SA-miRNA that regulates the senescence of MVECs

First, we analyzed miRNA levels using qRT-PCR in serially passaged MVECs, confirming that the expression of miR-214-5p was increased in senescent MVECs (Fig. 2A). Next, we measured senescence via the SA-β-gal assay (Fig. 2B) and SPiDER-β-gal assay (Fig. 2C) after transfection of miR-214-5p into young cells or an inhibitor of miR-214-5p into pre-senescent MVECs. Both results showed that the inhibition of miR-214-5p significantly delayed cellular senescence. Tube formation assays measure the ability of ECs to form capillary-like structures and are one of the most widely used *in vitro* assays to model the reorganization of angiogenesis. Tube formation assay showed that miR-214-5p reduced the angiogenic activity of young MVECs, whereas an inhibitor of miR-214-5p improved the tube-forming activity of senescent MVECs (Fig. 2D). These results suggested that miR-214-5p is a SA-miRNA that regulates MVEC senescence.

3.3. miR-214-5p regulates the expression of JAG1 in MVECs

To establish the mechanism by which miR-214-5p induces cellular senescence, we explored target genes containing the miR-214-5p seed sequence in the 3′-UTR using TargetScan v8.0, an miRNA target prediction platform [30]. The 3′-UTR sequences aligned to the miR-214-5p sequence are shown in Supplementary Table S4. Six candidate target genes were selected, and their expressions were analyzed by qRT-PCR (Fig. 3A). Among them, KLF5 and JAG1 mRNA levels decreased in serially passaged MVECs, which coincided with their protein levels (Fig. 3B). However, ectopic introduction of miR-214-5p or its inhibitor changed only the expression of JAG1, but not KLF5 (Fig. 3C), suggesting that KLF5 is not regulated by miR-214-5p in MVECs. Because it has been
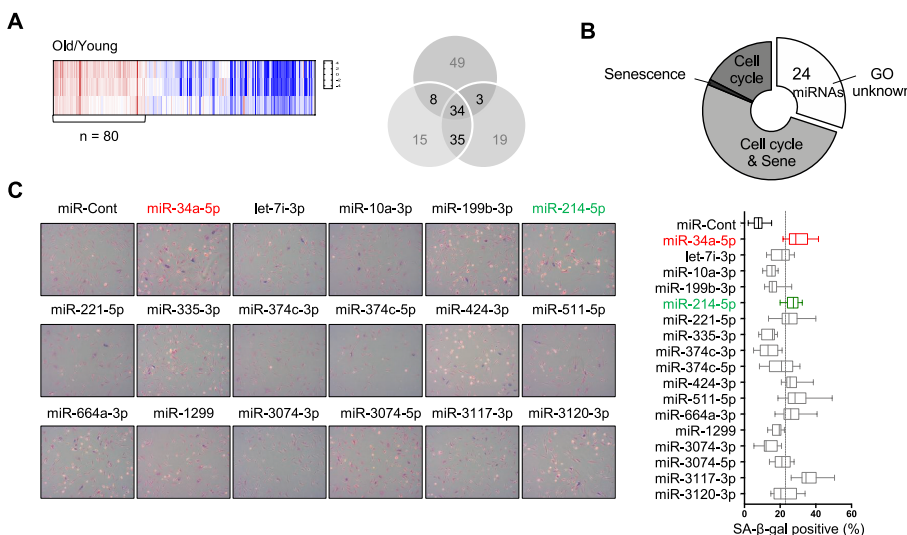

Fig. 1. Screening of SA-miRNAs in MVECs. (A) Differentially expressed miRNAs in senescent MVECs. A heatmap (left) shows the expression of miRNAs and eighty miRNAs were selected with 1.3 or more-fold induction in at least two old samples. The number in the Venn diagram represents the miRNAs induced in each senescent MVEC sample (right). (B) Gene Ontology analysis of selected SA-miRNA candidates. miRNAs known to regulate cell cycle or senescence were excluded. (C) SA-β-gal staining of MVECs after transfection with miRNA mimic. Representative images are shown on the left, and statistical analysis of three independent experiments is shown on the right. miR-Cont represents a negative control, and miR-34a-5p (red) is a positive control for senescence-inducing miRNAs. miR-214-5p, selected for further analysis, is shown in green.
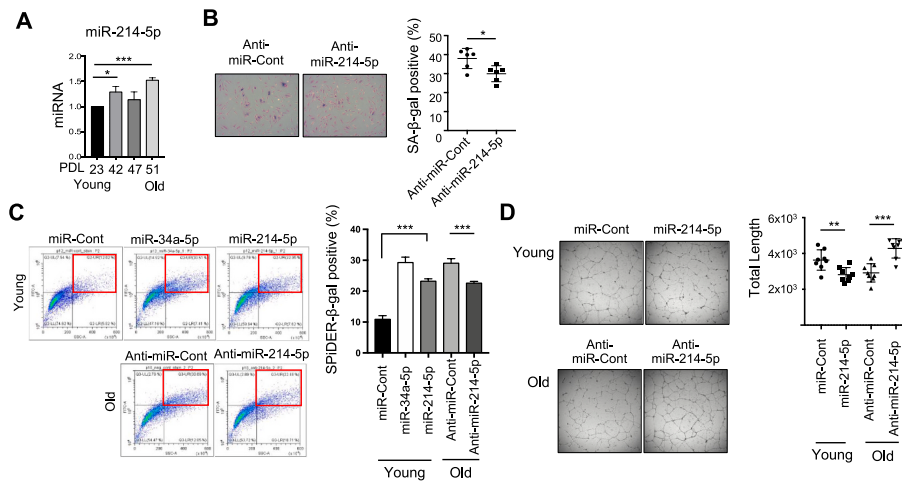
Fig. 2. miR-214-5p is a SA-miRNA and regulates the senescence of MVECs. (A) Expression of miR-214-5p was analyzed by qRT-PCR in serially passaged MVECs. (B) Old MVECs were transfected with an inhibitor of miR-214-5p and stained for SA-β-gal. The proportion of SA-β-gal positive cells decreased compared with the control. (C) Senescence was quantitatively measured via SPiDER-β-gal staining and flow cytometry. Young MVECs were treated with miR-214-5p mimic, and old MVECs were treated with miR-214-5p inhibitor. The proportion of SPiDER-β-gal positive cells was statistically compared to each control on the right. (D) The effect of miR-214-5p on the angiogenic activity of MVECs was analyzed via *in vitro* tube formation assays. The total length of tube-like structures in a field was compared to the control on the right.
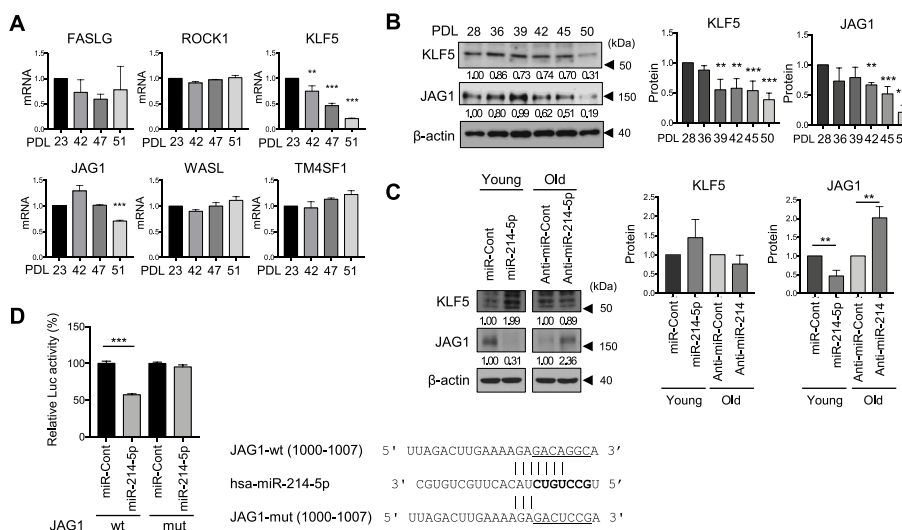

Fig. 3. Identification of miR-214-5p target gene. (A) The expression of candidate target genes of miR-214-5p was analyzed by qRT-PCR in serially passaged MVECs. (B) KLF5 and JAG1 protein levels were determined via immunoblotting analysis in serially passaged MVECs. (C) KLF5 and JAG1 protein levels were determined in young MVECs treated with miR-214-5p mimic and old MVECs treated with miR-214-5p inhibitor. (D) Luciferase activities were measured after co-transfection with the luciferase reporter gene and a miR-214-5p mimic into 293T cells. The luciferase reporter gene was fused with 3′-UTR sequences of a wild-type JAG1 (wt) or a mutated sequence (mut).

reported that JAG1 expression decreases with age in the basal layer of the epidermis [31], we speculated that senescence-associated induction of miR-214-5p could lead to cellular senescence through down-regulation of JAG1. The target specificity of miR-214-5p for JAG1 was confirmed using a luciferase (Luc) reporter assay. When the Luc reporter gene fused with the 3′-UTR sequence of JAG1 was co-transfected with an miR-214-5p mimic, Luc activity decreased, which was abolished when the miR-214-5p seed sequence in the 3′-UTR was mutated (Fig. 3D).

3.4. JAG1 silencing induced premature senescence in MVECs

Since we identified JAG1 as a specific target of miR-214-5p, we investigated whether the depletion of JAG1 is a significant factor that induces cellular senescence. We used a siRNA knockdown model to downregulate JAG1 expression in young MVECs. After siRNA transfection, the decrease in JAG1 expression was confirmed by immunoblotting (Fig. 4A). Under these experimental conditions, JAG1 silencing inhibited MVEC proliferation (Fig. 4B) and increased the proportion of SA-β-gal-positive cells (Fig. 4C). In addition, the knockdown of JAG1 reduced the angiogenic activity of MVECs (Fig. 4D). These results
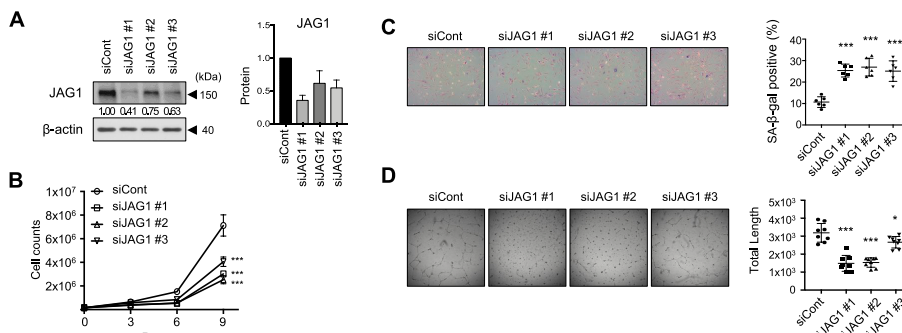

Fig. 4. JAG1 silencing induced premature senescence in MVECs. (A) Knockdown of JAG1 was confirmed via immunoblot analysis after transfection of three siRNAs designed to target JAG1. (B) Cell proliferation was determined by counting the viable cells after trypan blue staining every 3 d. (C) The effect of JAG1 silencing on cellular senescence was analyzed via SA-β-gal staining, and the proportion of SA-β-gal-positive cells is shown. (D) Angiogenic activity of JAG1-knockdown MVECs was analyzed via *in vitro* tube formation assay and quantified using Image J software.

suggest that downregulation of JAG1 by miR-214-5p could induce premature senescence of MVECs.

3.5. JAG1 overexpression delayed replicative senescence in MVECs

Next, we investigated whether ectopic overexpression of JAG1 delayed senescence progression. Retrovirus-mediated JAG1 overexpression was established in exponentially growing MVECs and serially passaged cells. JAG1 overexpression was confirmed by immunoblotting (Fig. 5A). Overexpression of JAG1 substantially increased the proliferative potential of MVECs compared with that of the control (Fig. 5B). In addition, JAG1 overexpression decreased the proportion of SA-β-gal-positive cells (Fig. 5C) and maintained a higher tube formation activity (Fig. 5D). Overexpression of JAG1 delayed replicative senescence in MVECs, which contrasted with the results of the downregulation of JAG1.

3.6. miR-214-5p induces cellular senescence by inhibiting JAG1-Notch signaling

JAG1, abundantly expressed in ECs, acts as a ligand for Notch signaling and activates the transcription of downstream genes *HES1* and *HEY1* [21,32]. To determine whether miR-214-5p-mediated cellular senescence occurred owing to the inhibition of JAG1-Notch signaling, we first investigated the expression of HES1 and HEY1 during senescence. In serially passaged MVECs, transcriptions of HES1 and HEY1 were decreased (Fig. 6A), and their protein levels were decreased (Fig. 6B), which is consistent with the results of miR-214-5p transfection (Fig. 6C and D). Furthermore, silencing of HES1 and HEY1 with siRNA (Fig. 6E) induced premature senescence in young MVECs (Fig. 6F). These results suggest that miR-214-5p induces cellular senescence through inhibition of JAG1-Notch signaling.

4. Discussion

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression. A single miRNA with a unique seed sequence can target hundreds of mRNAs, often simultaneously regulating an entire network or pathway [33]. In most cases, miRNAs control target gene expression by interacting with the 3′-UTR of mRNA, degrading the mRNA, and thereby inhibiting translation [34]. miRNAs are involved in various biological processes [35], such as senescence [18,19,36], and the abnormal expression of miRNAs is associated with many diseases [37,38]. Several studies have profiled many miRNAs that show increased or decreased expression during senescence [15–17]. For example, the expression of miR-519, a tumor suppressor miRNA, is increased in replicative senescent cells and can trigger senescence when overexpressed in young fibroblasts or HeLa cancer cells [17]. Many studies have identified SA-miRNAs targeting the p53-p21 axis and the p16-pRb pathway, which are central to senescence and cell cycle arrest mechanisms [39]. miR-152, which is increased in senescent cells, has been reported to reduce cellular adhesion and induce senescence by

inhibiting integrin α5 in human dermal fibroblasts [40]. Based on these reports, we screened novel SA-miRNAs in MVECs and attempted to elucidate the senescence-inducing mechanisms of miRNAs. After investigating for miRNAs that increase in old ECs (Fig. 1A), we excluded those whose gene ontology (GO) was associated with the cell cycle or senescence (Fig. 1B). Finally, we introduced 16 miRNA mimics of unknown GO into proliferating MVECs and performed SA-β-gal assay (Fig. 1C). After the screening of SA-miRNAs, we confirmed the expression and the senescence-regulating activity of these miRNAs (data not shown) from which we identified miR-664a-3p and miR-214-5p as SA-miRNAs, and further studied miR-214-5p to reveal its target gene and molecular mechanism in MVECs. We believe this study can be a very representative example that shows miRNA-mediated cellular senescence of ECs.

We first confirmed that miR-214-5p is SA-miRNA that regulates the angiogenic activity of MVECs, a typical phenotype of endothelial cells (Fig. 2). In various cancers, miR-214-5p inhibits proliferation, migration, and invasion [41–43]. Conversely, low miR-214-5p expression is associated with aggressive features in anaplastic large-cell lymphoma [44]. In addition, miR-214-5p inhibits the proliferation and migration of abnormal trophoblasts [45]. Although miRNA-214 has been reported to modulate senescence of vascular smooth muscle cells in carotid artery stenosis [46], the underlying mechanism has not yet been reported. Therefore, to determine the senescence-regulating mechanism of miR-214-5p, we searched for the target gene of miR-214-5p. Six target candidates were selected through sequence- and literature-based searches, but only KLF5 and JAG1 gene expression decreased in serially passaged MVECs (Fig. 3A). However, ectopic introduction of miR-214-5p or its inhibitor changed only JAG1 expression but not KLF5 in MVECs (Fig. 3C); therefore, we concluded that JAG1 is the target of miR-214-5p, whose specificity was supported by the Luc reporter assay (Fig. 3D).

JAG1, that is abundantly expressed in ECs, acts as a ligand for Notch signaling and regulates angiogenesis and vasculature development [21–23]. JAG1 expression is reduced in aged epidermis, and the interaction between JAG1 and Notch1 receptor prevents the accumulation of senescent cells [31]. Therefore, we investigated whether JAG1 controls senescence through Notch signaling in MVECs. JAG1 silencing induced premature senescence in proliferating cells (Fig. 4) and ectopic expression of JAG1 delayed replicative senescence in MVECs (Fig. 5). Furthermore, the expressions of HES1 and HEY1, key downstream target genes of JAG1-Notch signaling, were decreased in senescent cells and were regulated by miR-214-5p. In addition, knockdown of HES1 or HEY1 caused premature senescence of MVECs (Fig. 6F). These results suggest that downregulation of JAG1 by miR-214-5p induced senescence of MVECs through the depletion of HES1 and HEY1.

HES1 and HEY1 are transcriptional repressors that regulate cell cycle progression by inhibiting gene expression [47]. HES1 represses the transcription of Phosphatase and Tensin Homolog (PTEN), inhibiting PI3K/Akt-mediated phosphorylation and GSK3β [48]. HES1 promotes cell cycle progression by transcriptional repression of the cyclin-dependent kinase inhibitor p27 [49]. Without HES1, the levels of
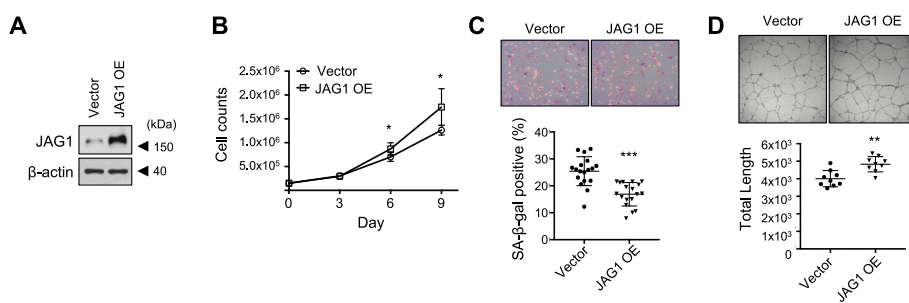

Fig. 5. JAG1 overexpression delayed replicative senescence in MVECs. (A) Ectopic JAG1 was introduced in MVECs (PDL30) and passaged serially to PDL45. Overexpression of JAG1 was confirmed via immunoblot analysis. (B) Cell proliferation was determined by counting the viable cells after trypan blue staining every 3 d. (C) Senescence in MVECs (PDL41) was determined via SA-β-gal staining and the proportion of SA-β-gal-positive cells was compared to control. (D) Angiogenic activity of JAG1-overexpressed MVECs was analyzed via *in vitro* tube formation assay and quantified.
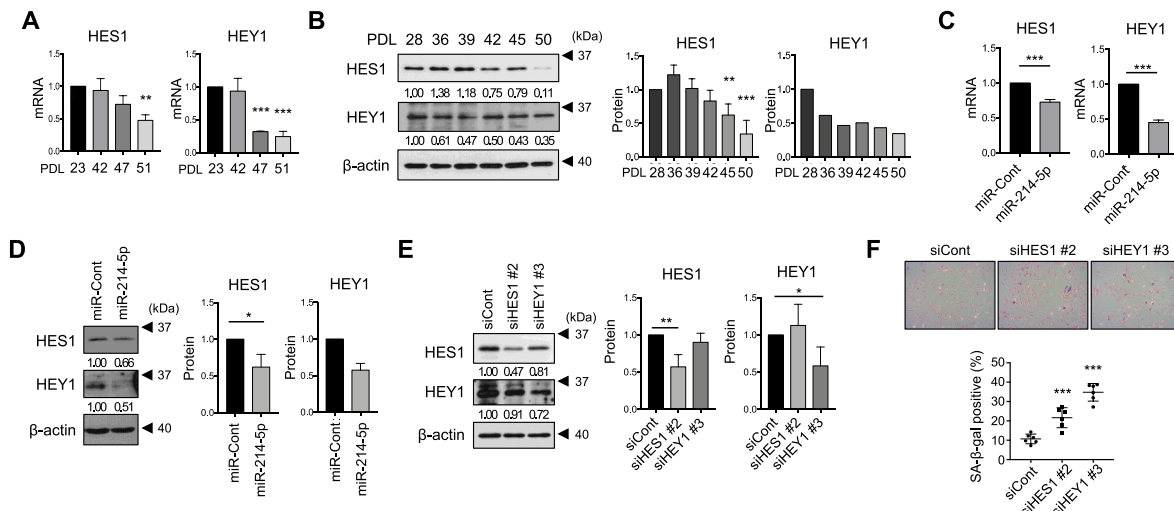
Fig. 6. miR-214-5p induces senescence through downregulation of JAG1-Notch signaling. Expressions of HES1 and HEY1 were analyzed via qRT-PCR (A) and immunoblotting (B) in serially passaged MVECs. In miR-214-5p-transfected MVECs, the expressions of HES1 and HEY1 were analyzed by qRT-PCR (C) and immunoblotting (D). Knockdown of HES1 and HEY1 using siRNA was confirmed by immunoblotting analysis (E) and HES1- and HEY1-downregulated MVECs were stained for SA-β-gal (F). The proportion of SA-β-gal positive cells was compared to control in the right.

cyclin-dependent kinase inhibitors p27 and p57 increase significantly, leading to impaired cell cycle progression [50]. Similarly, HEY1 is an effective repressor of p57 [51]. In addition, Notch activates p53 via HEY1-mediated inhibition of the key p53 inhibitor MDM2 [52]. Therefore, miR-214-5p-mediated downregulation of HES1 and HEY1 could induce cell cycle arrest at the G1 phase, leading to senescence of MVECs.

5. Conclusions

In this study, we characterized differentially expressed miRNAs during replicative senescence in microvascular endothelial cells. We identified miR-214-5p as a senescence-associated miRNA, and JAG1 as a prime target of miR-214-5p induced during cellular senescence. As miR-214-5p-JAG1-Notch-HEY1/HES1 signaling plays a crucial role in senescence, targeting miR-214-5p could be a potential strategy to delay vascular aging and present a unique therapeutic opportunity to overcome the detrimental effects of senescence such as CVD and other age-related diseases.

CRediT authorship contribution statement

Hye-ram Jo: Investigation, Validation, Formal analysis, Visualization, Writing – original draft. **Jiwon Hwang:** Investigation, Validation. **Jae-Hoon Jeong:** Conceptualization, Supervision, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2023.05.002.

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