Rapamycin-induced miR-30a downregulation inhibits senescence of VSMCs by targeting Beclin1

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Abstract. Vascular senescence is considered to be an independent risk factor for cardiovascular diseases. The present study aimed to investigate the effects of rapamycin on miR-30a and its relationship with autophagy and senescence in vascular smooth muscle cells (VSMCs). Young and aging VSMCs were treated with rapamycin or transfected with miR-30a mimics. Measurement of cellular senescence was conducted using senescence-associated (SA)-β-Galactosidase (gal) staining. Dual luciferase reporter assay was used to confirm binding for miR-30a and Beclin1. The expression levels of miR-30a and Beclin1 were determined with reverse transcription-quantitative polymerase chain reaction analysis. Autophagy-related protein levels were determined using immunofluorescence or western blot assays. The results demonstrated that rapamycin treatment significantly decreased miR-30a expression and increased Beclin1 expression in both young and aging cells, as well as promoted autophagy in VSMCs. In addition, rapamycin inhibited senescence in VSMCs and could also alleviate the aging VSMC cycle arrest. Dual luciferase reporter assay confirmed that miR-30a could directly bind the 3'untranslated region of Beclin1 and inhibit its expression. Furthermore, miR-30a inhibited autophagy and promoted senescence of VSMCs. In conclusion, the present results indicated that rapamycin could inhibit the senescence of VSMCs by downregulating miR-30a, which resulted in upregulation of Beclin1 and activation of autophagy. The current study is the first to demonstrate an inhibitory role of rapamycin on VSMC senescence and might provide novel insights and potential new molecular targets in senescence treatment.

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Introduction

Cardiovascular disease is one of the major threats to human life and health, and vascular senescence is an important cause of its occurrence. Vascular senescence is also considered to be an independent risk factor for cardiovascular diseases (1). Senescence, which is thought to be irreversible, is considered to contribute to alteration in cell function, morphology, and gene expression (2), and thus has an important role in diseases, including type 2 diabetes, cancer, neurodegeneration, and age-associated cardiovascular diseases, such as atherosclerosis (3). It is thought that vascular smooth muscle cells (VSMCs) have a key role in vascular aging and contribute to the initiation and progression of atherosclerosis (4,5). Since no physiological stimuli are known currently to cause senescent cells to re-enter the cell cycle, the treatment of senescence remains a challenge (6). Thus, an in-depth understanding of the molecular mechanisms of senescence and of potential molecular targets for drug design is an important research direction for the treatment of senescence.

Along with age and cancer (5), autophagy is considered to be another factor affecting senescence (7). Studies have demonstrated that autophagy has a crucial role in the regulation of cellular senescence, through degradation of aggregate-prone proteins and damaged organelles (8). The autophagy process is associated with many proteins and signaling pathways, such as the autophagy proteins autophagy-related gene 6 (Atg6)/Beclin1, and the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways (9-11). Studies have demonstrated that the inhibition of mTOR promotes longevity and expression of autophagy biomarkers, and that the complex formed by Atg6/Beclin1 and phosphoinositide 3-kinase (PI3K) was responsible for autophagosome formation (7,9,12). However, the relation of autophagy-related signaling with senescence requires further study.

Rapamycin, an antibiotic that stimulates autophagy by inhibition of mTOR signaling (13), is thought to also influence the aging process (14). As previously reported, rapamycin suppresses replicative senescence in rodent embryonic cells (15), and is involved in regulation of cell senescence by different mechanisms (16). A previous study revealed that rapamycin treatment in mice promotes healthy longevity

by targeting aging, leading to increased lifespan and health span (14). Additionally, it was reported that microRNA (miR)-30a, also known as an aged-related miRNA (17,18), regulates rapamycin-induced autophagy in cancer cells by targeting Beclin1 (19). Furthermore, rapamycin also partly decreases the effect of miR-30a on osteosarcoma cell apoptosis, by activating autophagy through regulating Beclin1 and microtubule-associated protein 1 light chain 3 β (LC3B) (20). However, deeper insights between rapamycin and miR-30a still lack in vascular senescence.

To date, no study has focused on whether rapamycin could regulate vascular senescence by modulating miR-30a and autophagy. The present study aimed to investigate the effects of rapamycin on miR-30a, as well as on autophagy and senescence, in VSMCs.

Materials and methods

Cell culture and treatment. VSMC isolation and cell culture have been previously described (21). The present study was approved by the Ethics Committee of the Department of Laboratory Animal Science, Central South University (Changsha, China) prior to the experiments. Briefly, VSMCs were isolated from the thoracic aorta of SD rats. A total of 6 male Sprague-Dawley rats aged 5-6 weeks and weighting 160-220 g were purchased from Human SJA Laboratory Animal Co., Ltd. (Changsha, China). All animals were housed in micro-isolator cages with free access to food and water in a light-controlled room under a 12/12 h light/dark cycle and controlled temperature (23-25˚C). Aortic VSMCs were then cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Merck KGaA, Darmstradt, Germany) supplemented with 10% Gibco fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 μg/ml penicillin-streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C and 5% CO_2. VSMCs of passage 5 (young) and passage 15 (aging) at 70-90% confluence were used. All cells were divided into four groups: Young VSMCs, young VSMCs with rapamycin, aging VSMCs, and aging VSMCs with rapamycin. For the groups treated with rapamycin, VSMCs were treated with 20 nM rapamycin (Sigma-Aldrich; Merck KGaA) for 12 h. The untreated cells were used as controls.

Cell transfection. The miR-30a mimics and negative control (NC) were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences of miR-30a mimics and miR-NC were 5'-UGUAAACAUCCUCGACUGGAAG-3' and 5'-UUCUCCGAACGUGUCACGUTT-3', respectively. The aging cells were pre-transfected with miR-30a mimics or miR-NC with a final concentration of 50 nM for 48 h, prior to treatment with rapamycin for 12 h. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect oligonucleotides and constructs into cell lines, according to the manufacturer's instructions. The infection efficiency was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or western blotting 48 h post-transfection.

Cell cycle analysis. For cell cycle analysis, cells were fixed with 70% cold ethanol overnight at 4˚C, washed with PBS, and then stained with 5 mg/ml propidium iodide in the presence of RNase A (10 μg/ml) for 30 min. Cell cycle phase distribution was analyzed by flow cytometry as reported elsewhere (22).

Senescence-associated (SA)-β-Galactosidase (gal) staining. Measurement of cellular senescence was conducted by SA-β-gal staining. Briefly, cells were fixed with 4% formaldehyde for 15 min at room temperature, washed with PBS, and then incubated at 37˚C overnight in SA-β-gal staining solution (1 mg/ml of X-gal; 40 mmol/l citric acid/sodium phosphate buffer, pH 6.0; 5 mmol/l potassium ferrocyanide; 5 mmol/l potassium ferricyanide; 150 mmol/lNaCl; and 2 mmol/l $MgCl_2$). Next day, the slides were washed twice in PBS, mounted in glycerol, and observed in five optical fields per sample using a light microscope.

Immunofluorescence. Immunofluorescence was conducted to evaluate the expression of LC3B. The cells were fixed with 4% formaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature and then incubated with anti-LC3B rabbit antibodies (cat. no. ab48394, 1:200; Abcam, Cambridge, MA, USA) overnight at 4˚C. The cells were then incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG antibodies (cat. no. ab150080; 1:1,000; Abcam) for 1 h at room temperature. The nuclei of ells were then counterstained with DAPI for 10 min at room temperature. A Leica TCS-SP laser scanning confocal microscope was used to capture the photomicrographs in five optical fields per sample.

Dual luciferase reporter assay. For the dual luciferase reporter assay, the wild type (WT) or a mutant (MUT) 3'-untranslated region (UTR) sequence of Beclin1 was amplified and subcloned into the pGL3-basic luciferase vector (Promega Corporation, Madison, WI, USA). VSMCs were co-transfected with either the WT or MUT 3'-UTR sequence of Beclin1 (1 μg), together with 25 nM miR-30a mimics or negative control. After 48 h of transfection, relative luciferase activity was measured with the Bright-Glo Luciferase Assay System (Promega Corporation).

RT-qPCR. The expression levels of miR-30a and Beclin1 were determined by RT-qPCR. Briefly, total RNA was extracted from VSMCs with TRIzol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. The PrimeScriptOne-Step RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to convert RNA into cDNA. PCR reactions were conducted using SYBR®-Green PCR Master Mix (Takara Biotechnology Co., Ltd.) in an ABI7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers sequences for Beclin1 were: Forward 5'-GTG CTCCTGTGGAATGGAAT-3' and reverse 5'-TGCAAC ACAGTCCAGAAAAGC-3'. The primers for miR-30a were purchased from Takara Biotechnology Co., Ltd., and were: Forward 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACCTTCCA-3' and reverse 5'-GGCGTG TAAACATCCTCGAC-3. The primers sequences for GAPDH were: Forward 5'-GGATTTGGTCGTATTGGG-3' and reverse 5'-GGAAGATGGTGATGGGATT-3'. The primers sequences for U6 were: Forward 5'-ATTGGAACGATACAGAGAAG ATT-3' and reverse 5'-GGAACGCTTCACGAATTTG-3'.

Relative fold changes in mRNA expression were calculated using the $2^{-\Delta\Delta Cq}$ formula (23). GAPDH and U6 served as internal controls for mRNA and miRNA respectively.

Western blot analysis. The protein levels of LC3, p62, Beclin1, mTOR, phosphorylated (p-) mTOR, p53, p21, p16 and SA-β-gal were determined by western blotting. Briefly, total protein was extracted from VSMCs using a RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) and was quantitated with protein assay reagent from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Then equal amount of samples (30 μg) were loaded on 10% SDS-PAGE, followed by transfer to PVDF membranes. After blocking with 5% non-fat milk at room temperature for 1 h, the membranes were then incubated with primary antibodies (all purchased from Abcam) targeting LC3B (cat. no. ab48394; 2 μg/ml), p62 (cat. no. ab56416; 5 μg/ml), Beclin1 (cat. no. ab62557; 2 μg/ml), p-Beclin1 (cat. no. ab183335; 1:250), mTOR (cat. no. ab2732; 1:2,000), p-mTOR (S2448; cat. no. ab109268; 1:100), p-ribosomal protein S6 kinase B1 (S6K1; cat. no. ab2571; 1:500), p-eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; cat. no. ab75767; 1:1,000), p53 (cat. no. ab131442; 1:1,000), p21 (cat. no. ab109520; 1:1,000), p16 (cat. no. ab51243; 1:10,000), SA-β-gal (cat. no. ab9361; 0.5 μg/ml) and GAPDH (cat. no. ab8245; 1:500) at 4˚C overnight. Subsequently, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG; cat. no. ab6940 and goat anti-mouse IgG; cat. no. ab97035; both 1:500) at 37˚C for 45 min. The target bands were then developed using Super Signal West Pico Chemiluminescent Substrate kit (Pierce; Thermo Fisher Scientific, Inc.) and analyzed by Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). GAPDH served as an internal control.

Statistical analysis. All experiments were performed at least three independent times in triplicate. Data were presented as mean ± standard deviation. Statistical analyses were performed with Graphpad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons were conducted using one-way analysis of variance followed by Tukey post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rapamycin inhibits senescence in VSMCs and alleviates cell cycle arrest. SA-β-gal staining was conducted to examine the effect of rapamycin on senescence of VSMCs. As illustrated in Fig. 1A and B, the ratio of SA-β-gal-positive cells was significantly higher in aging cells compared with young cells ($P<0.01$). However, following rapamycin treatment, the ratio of SA-β-gal-positive cells significantly decreased in aging cells (Fig. 1B), indicating that rapamycin could inhibit senescence in VSMCs.

Flow cytometry analysis revealed that G1 arrest occurred in aging cells and this cell cycle arrest was reduced when cells were treated with rapamycin (Fig. 1C and D). Further analysis of p16, p21, p53 and SA-β-gal expression levels by western blotting demonstrated that rapamycin treatment reduced the increasing levels of senescence-related proteins in aging cells (Fig. 1E and F). These results suggested that rapamycin could inhibit senescence of VSMCs through inhibition of cell cycle arrest and senescence-related proteins.

Rapamycin inhibits miR-30a expression and promotes autophagy in VSMCs. To examine the effect of rapamycin on the expression of miR-30a and autophagy of VSMCs, the expression of miR-30a and Beclin1 was determined by RT-qPCR. The results demonstrated that in aging cells miR-30a was significantly upregulated while Beclin1 was significantly downregulated compared with young cells ($P<0.01$; Fig. 2A and B), indicating that both miR-30a and Beclin1 might be associated with senescence in VSMCs. However, when treated with rapamycin, miR-30a was significantly downregulated in both young and aging cells ($P<0.05$; Fig. 2A). Additionally, the expression of Beclin1 was significantly upregulated in both young and aging cells following rapamycin treatment ($P<0.05$; Fig. 2B). These results clearly demonstrated that rapamycin inhibited miR-30a expression while it induced Beclin1 expression.

To further investigate the effect of rapamycin on autophagy in VSMCs, the expression of LC3, Beclin1, p62, mTOR and p-mTOR was determined by western blotting. As illustrated in Fig. 2C and D, the ratio of LC3-II/LC3-I and the expression of Beclin1 and p-Beclin1 were significantly downregulated, while the expression of p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1 was significantly upregulated in aging cells compared with young cells ($P<0.05$). These findings indicate that autophagy-related proteins and mTOR signaling were associated with cell aging. Similarly, when treated with rapamycin, the ratio of LC3-II/LC3-I and the expression of both Beclin1 and p-Beclin1 were all significantly upregulated, while the expression of p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1 significantly downregulated (Fig. 2C and D). Immunofluorescence analysis of LC3B protein expression demonstrated the same results (Fig. 2E). In both young and aging cells, when treated with rapamycin, the expression of LC3B markedly increased compared with the untreated cells (Fig. 2E), suggesting that rapamycin might promote autophagy of VSMCs to influence the cell aging process.

miR-30a directly downregulates Beclin1. To investigate the relationship between miR-30a and Beclin1, aging cells were treated with rapamycin or miR-30a mimics and the expression of miR-30a and Beclin1 was determined. The results demonstrated that expression of miR-30a was significantly upregulated following transfection with miR-30a mimics and significantly downregulated following rapamycin treatment ($P<0.01$; Fig. 3A). This indicated that the transfection of miR-30a mimics was successful. When treated with miR-30a mimics, expression of Beclin1 significantly decreased in aging cells, and the rapamycin-induced upregulation of Beclin1 was significantly reversed at both the mRNA and protein levels ($P<0.01$; Fig. 3B-D), indicating that miR-30a could downregulate the expression of Beclin1.

To further examine the function of miR-30a on regulating Beclin1, a dual luciferase reporter assay was conducted. Bioinformatics analysis by software target

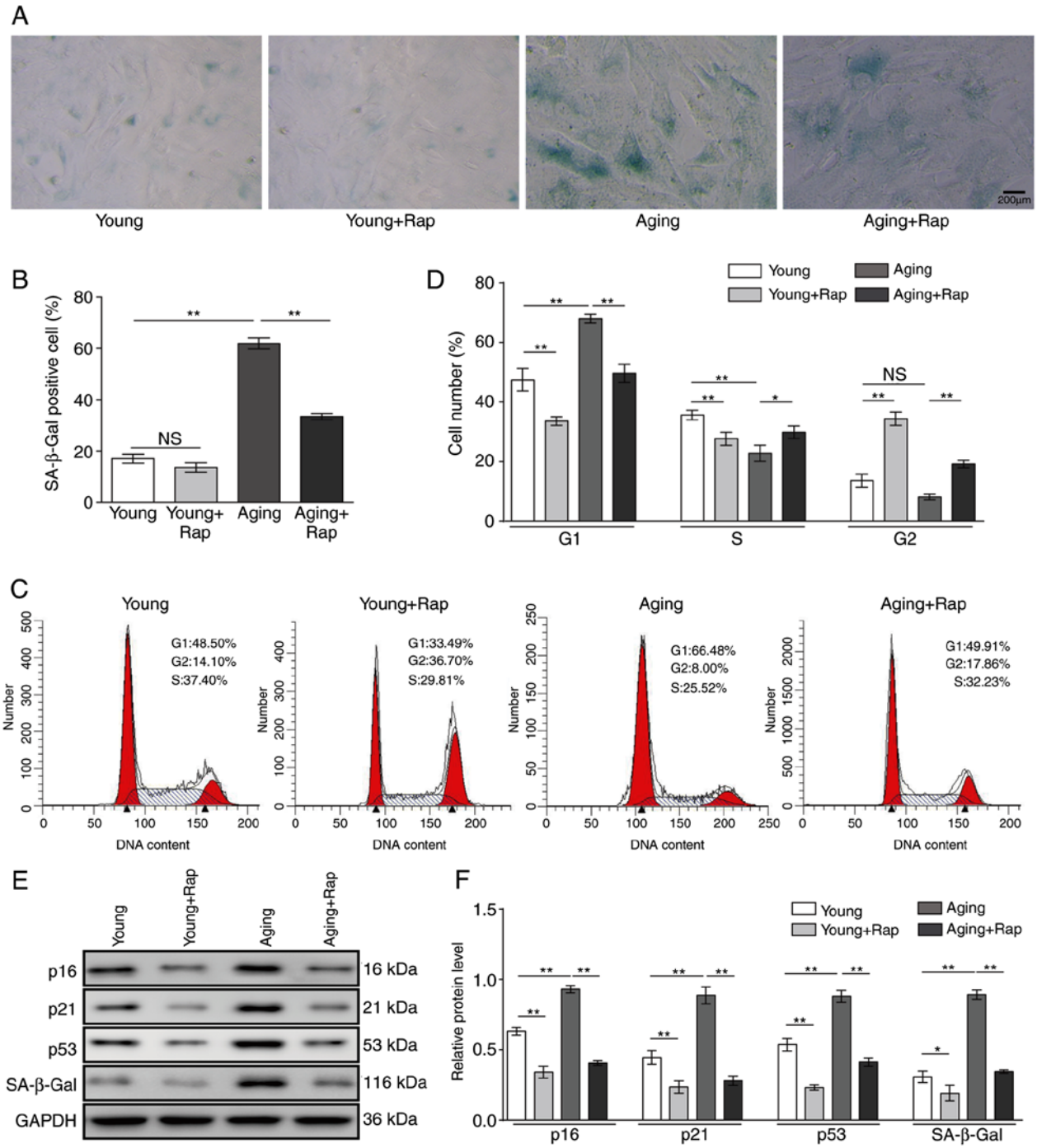

Figure 1. Rapamycin inhibits senescence in VSMCs and alleviates cell cycle arrest. Young and aging VSMCs were treated with 20 nM rapamycin for 12 h. (A) Cell senescence was measured by SA-β-gal staining. Representative images are shown. Scale bar, 200 μm. (B) SA-β-gal-positive cell rates in the different groups. (C) Representative plots and (D) quantification of flow cytometry analysis for cell cycle phase distribution. (E) Protein expression levels of p16, p21, p53 and SA-β-gal were determined by western blotting. Representative blots are shown. (F) Quantitative analysis of indicated proteins. Results are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by lines. VSMCs, vascular smooth muscle cells; SA-β-gal, senescence-associated-β-Galactosidase; Rap, rapamycin; ns, not significant.

scan 5.1 (http://www.targetscan.org) revealed that there is a potential miR-30a binding site on the 3'-UTR of Beclin1 in rats (Fig. 3E). Furthermore, the sequence of miR-30a was identical and the homology of Beclin1 was high in both rat and human, so the binding sites of miR-30a and Beclin1 were the same in rat and human (Fig. 3E). The results from the luciferase assay demonstrated that the relative luciferase activity in WT-Beclin1 was significantly downregulated by miR-30a mimics, while no significant change was observed with MUT-Beclin1 (Fig. 3F), further confirming that miR-30a directly bound to the Beclin1 3'-UTR.

Rapamycin alleviates senescence and cell cycle arrest in VSMCs by inhibiting miR-30a. At last, the present study investigated the effects of miR-30a on senescence and the cell cycle in VSMCs. Similar to the aforementioned results, the

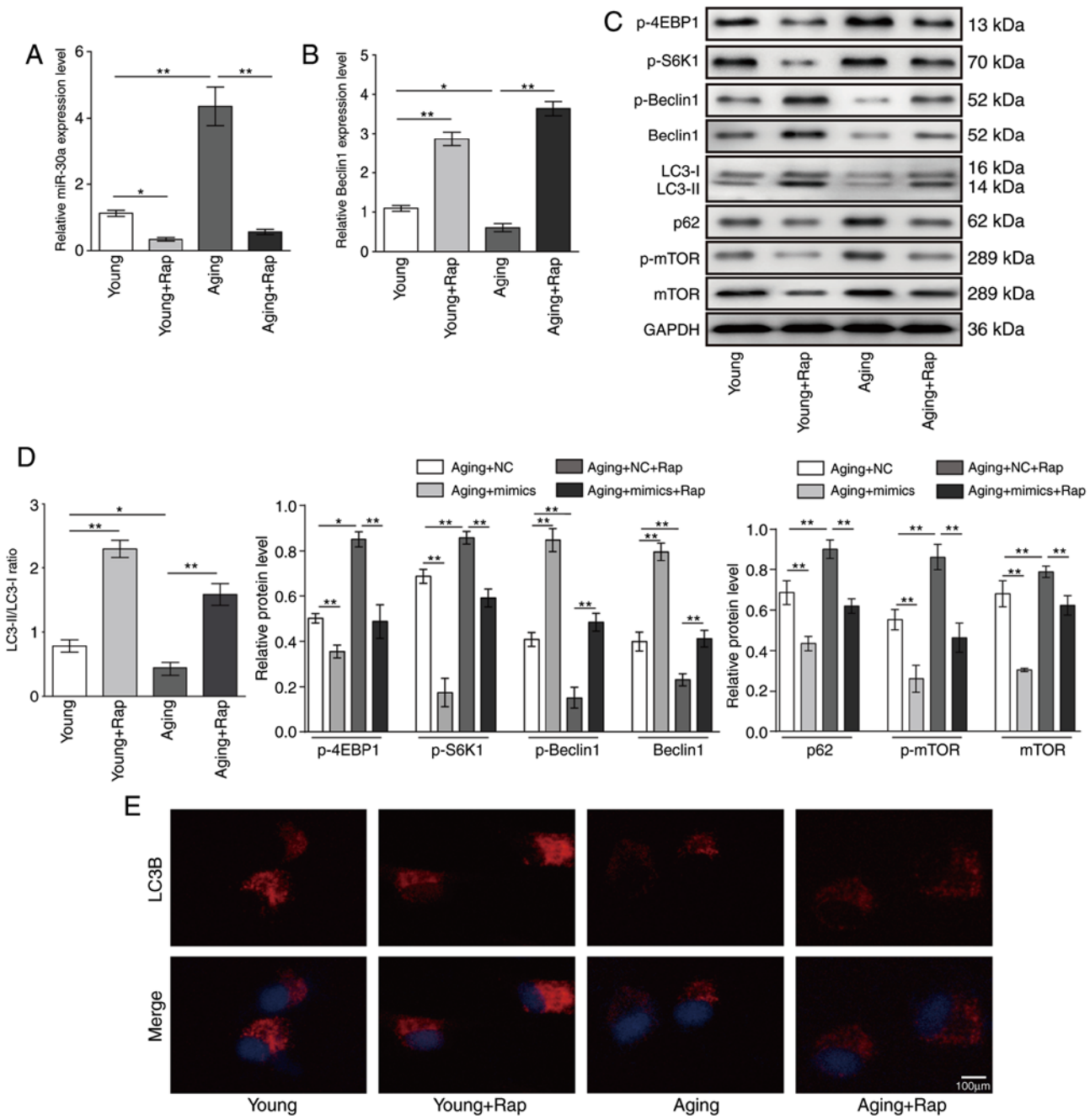

Figure 2. Rapamycin inhibits miR-30a expression and promotes autophagy in VSMCs. (A) Relative expression levels of miR-30a and (B) Beclin1 were determined by reverse transcription-quantitative polymerase chain reaction in young and aging VSMCs treated with 20 nM rapamycin for 12 h. (C) Representative blots of LC3, Beclin1, p-Beclin1, p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1 protein expression levels in young and aging VSMCs treated with 20 nM rapamycin for 12 h. (D) Quantitative analysis for LC3-II/LC3-I ratio, Beclin1, p62, mTOR and p-mTOR protein expression. (E) Immunofluorescence analysis for LC3B in young and aging VSMCs treated with 20 nM rapamycin for 12 h. Results are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by lines. VSMCs, vascular smooth muscle cells; LC3, microtubule-associated protein 1 light chain 3 β; p-, phosphorylated; mTOR, mammalian target of rapamycin; S6K1, ribosomal protein S6 kinase B1; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; Rap, rapamycin.

ratio of SA-β-gal positive cells was significantly upregulated when cells were transfected with miR-30a mimics (P<0.01; Fig. 4A and B). In addition, the rapamycin inhibitory effect on senescence of VSMCs was significantly reversed by overexpression of miR-30a (P<0.01; Fig. 4A and B). Notably, transfection reagent Lipofectamine 2000 alone had no significant effect on VSMC senescence (Fig. 4A and B).

Flow cytometry analysis revealed that G1 arrest was significantly promoted in aging cells following transfection with miR-30a mimics (Fig. 4C and D). Western blotting results demonstrated that overexpression of miR-30a could significantly increase the levels of the senescence-related proteins p16, p21, p53 and SA-β-gal (P<0.01; Fig. 4E and F). Furthermore, the rapamycin inhibition effects on both cell cycle arrest and senescence-related protein expression were reversed upon transfection with miR-30a mimics (P<0.05; Fig. 4E and F). These results suggested that miR-30a could promote senescence and cell cycle arrest in VSMCs, and that rapamycin might inhibit senescence of VSMCs through inhibition of miR-30a.

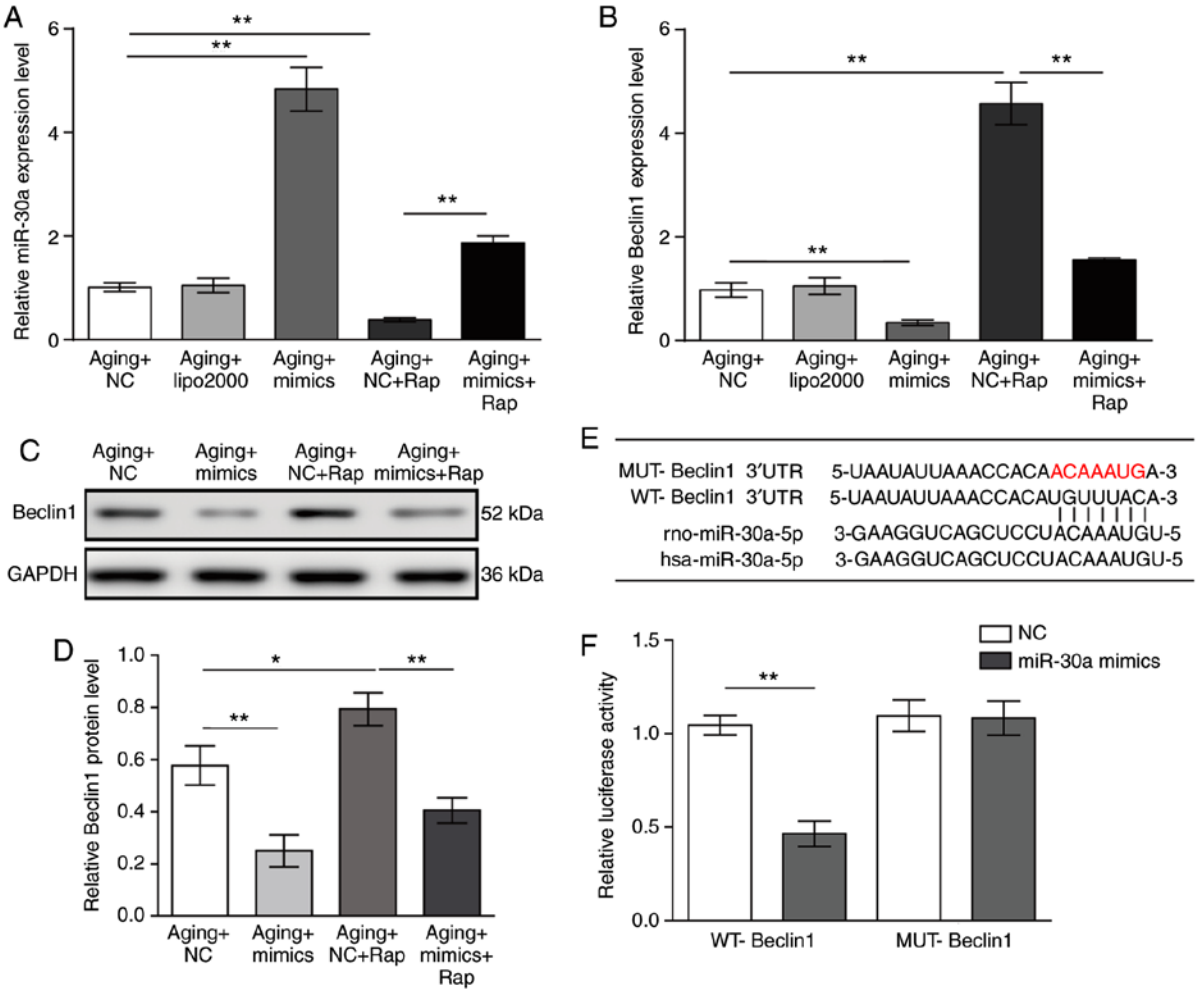

Figure 3. miR-30a directly downregulates Beclin1. (A) Relative expression levels of miR-30a and (B) Beclin1 were determined by reverse transcription-quantitative polymerase chain reaction in aging VSMCs transfected with miR-30a mimics or negative control for 48 h followed by 20 nM rapamycin for 12 h. (C) Representative blots and (D) quantification of Beclin1 protein expression levels in aging VSMCs transfected with miR-30a mimics or negative control for 48 h followed by 20 nM rapamycin for 12 h. (E) The predicted binding site of miR-30a on Beclin1 3'-UTR in both the rat and human genes. A mutant binding site was constructed and the red letters indicate mutated nucleotides. (F) Relative luciferase activity was evaluated. Results are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by lines. VSMCs, vascular smooth muscle cells; UTR, untranslated region; NC, negative control; Rap, rapamycin; WT, wild type; MUT, mutant.

Rapamycin promotes autophagy in VSMCs via inhibiting miR-30a. The effect of miR-30a on autophagy of VSMCs was then investigated by determining the protein expression of autophagy-related proteins LC3, Beclin1, p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1. As illustrated in Fig. 5A and B, the LC3-II/LC3-I ratio and Beclin1 expression levels were significantly downregulated, while the expression levels of p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1 were significantly upregulated in aging cells following transfection with miR-30a mimics, suggesting that miR-30a could inhibit autophagy of aging cells. Additionally, miR-30a mimics could significantly reverse the alteration of all autophagy-related proteins induced by rapamycin (Fig. 5A and B). Similar results were also observed by immunofluorescence analysis of LC3B expression. In both control cells and cells treated with rapamycin, when transfected with miR-30a mimics, the expression of LC3B markedly decreased (Fig. 5C). The present results fully demonstrated that miR-30a could inhibit autophagy of VSMCs and that rapamycin could induce autophagy of VSMCs through inhibition of miR-30a.

Discussion

In the present study, rapamycin treatment was demonstrated for the first time to upregulate Beclin1 and to activate autophagy by downregulating miR-30a, which further alleviated the senescence of VSMCs. The present study provided novel insights into the inhibitory effects of rapamycin on senescence of VSMCs, and may serve as a basis for a potential clinical application of rapamycin in treatment of senescence-related diseases.

Rapamycin could influence the senescence processes. As reported, rapamycin inhibits senescence of mouse hematopoietic stem cells (24), and modulates cell senescence and inflammation by different mechanisms (16). A previous study focused on the effect of rapamycin in aged mice and reported that rapamycin could enhance the resistance of aged mice to pneumococcal pneumonia through reduced cellular senescence (25). All the above results are consistent with the present findings that rapamycin could inhibit senescence. Additionally, the current study demonstrated that rapamycin could inhibit

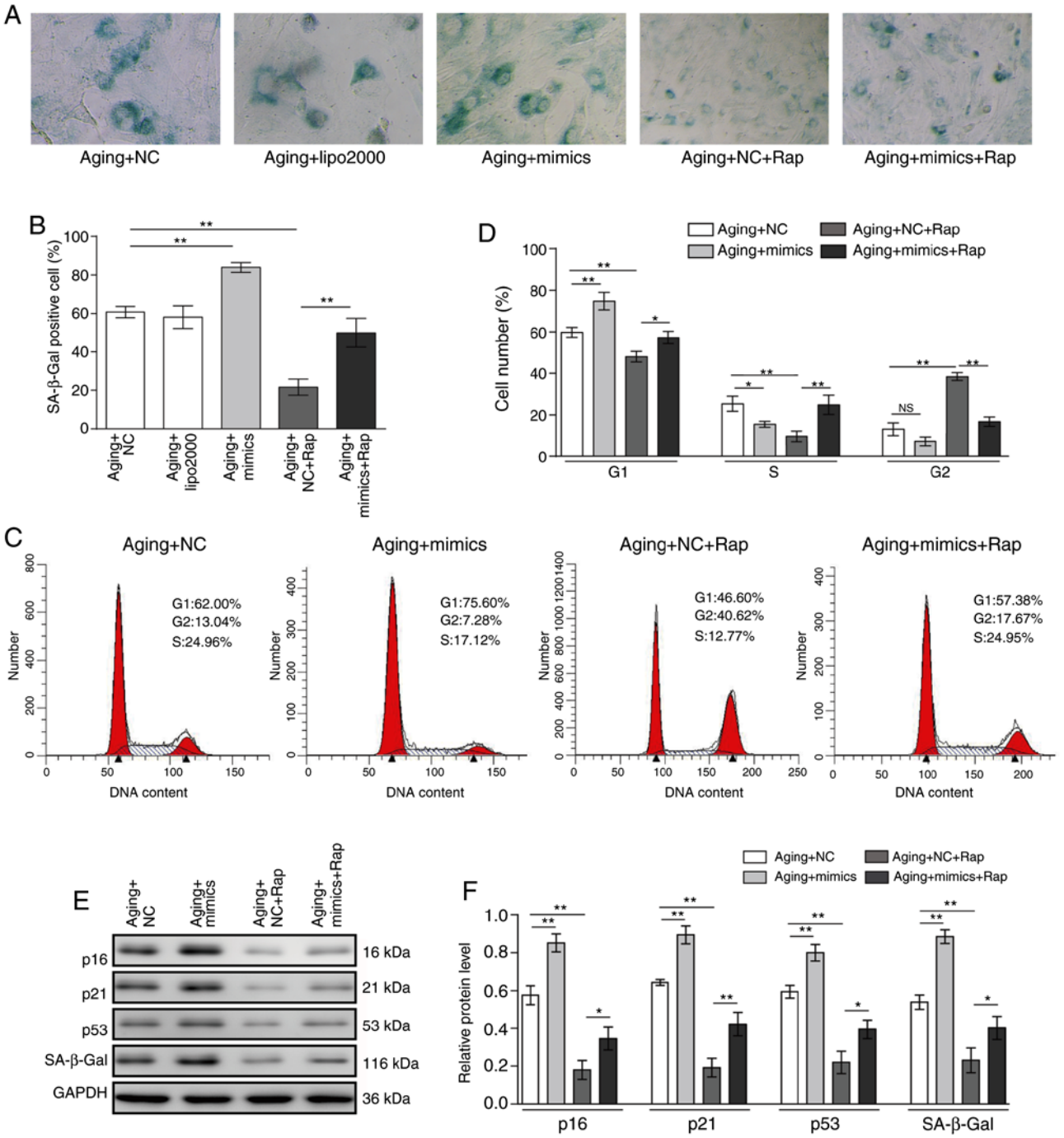

Figure 4. Rapamycin alleviates senescence and cell cycle arrest in VSMCs by inhibiting miR-30a. VSMCs were transfected with miR-30a mimics or negative control for 48 h, and then treated with 20 nM rapamycin for 12 h. (A) Senescence was determined by SA-β-gal staining. Representative images are shown. Scale bar, 300 μm. (B) SA-β-gal-positive cell rates in the different groups. (C) Representative plots and (D) quantification of flow cytometry analysis for cell cycle phase distribution. (E) Protein expression levels of p16, p21, p53 and SA-β-gal were determined by western blotting. Representative blots are shown. (F) Quantitative analysis of indicated proteins. Results are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by lines. VSMCs, vascular smooth muscle cells; SA-β-gal, senescence-associated-β-Galactosidase; NC, negative control; Rap, rapamycin; ns, not significant.

cell cycle arrest. A study on the relationship between rapamycin and normal aging demonstrated that rapamycin could block cell aging by inhibiting cell cycle arrest (26), which is consistent with the current findings. The p53, p21 and p16 proteins are generally considered to be associated with cell cycle arrest and the activation of p53/p21 signaling is thought to contribute to cell cycle arrest (27,28). In the present study, rapamycin was demonstrated to inhibit the expression of cell cycle-related proteins p53, p21 and p16, suggesting that rapamycin inhibited cell cycle arrest via repressing the p53/p21 signaling pathway. These results confirmed that rapamycin could alleviate senescence of VSMCs as well as inhibit cell cycle arrest.

It has been reported that rapamycin promotes autophagy in multiple cells or diseases, such as neural tissue damage, melanoma cells and stem cells (16,29). However, the effects of rapamycin on autophagy and related mechanisms in the process of vascular senescence remain unclear. As reported, the serine/threonine kinase unc-51 like autophagy activating

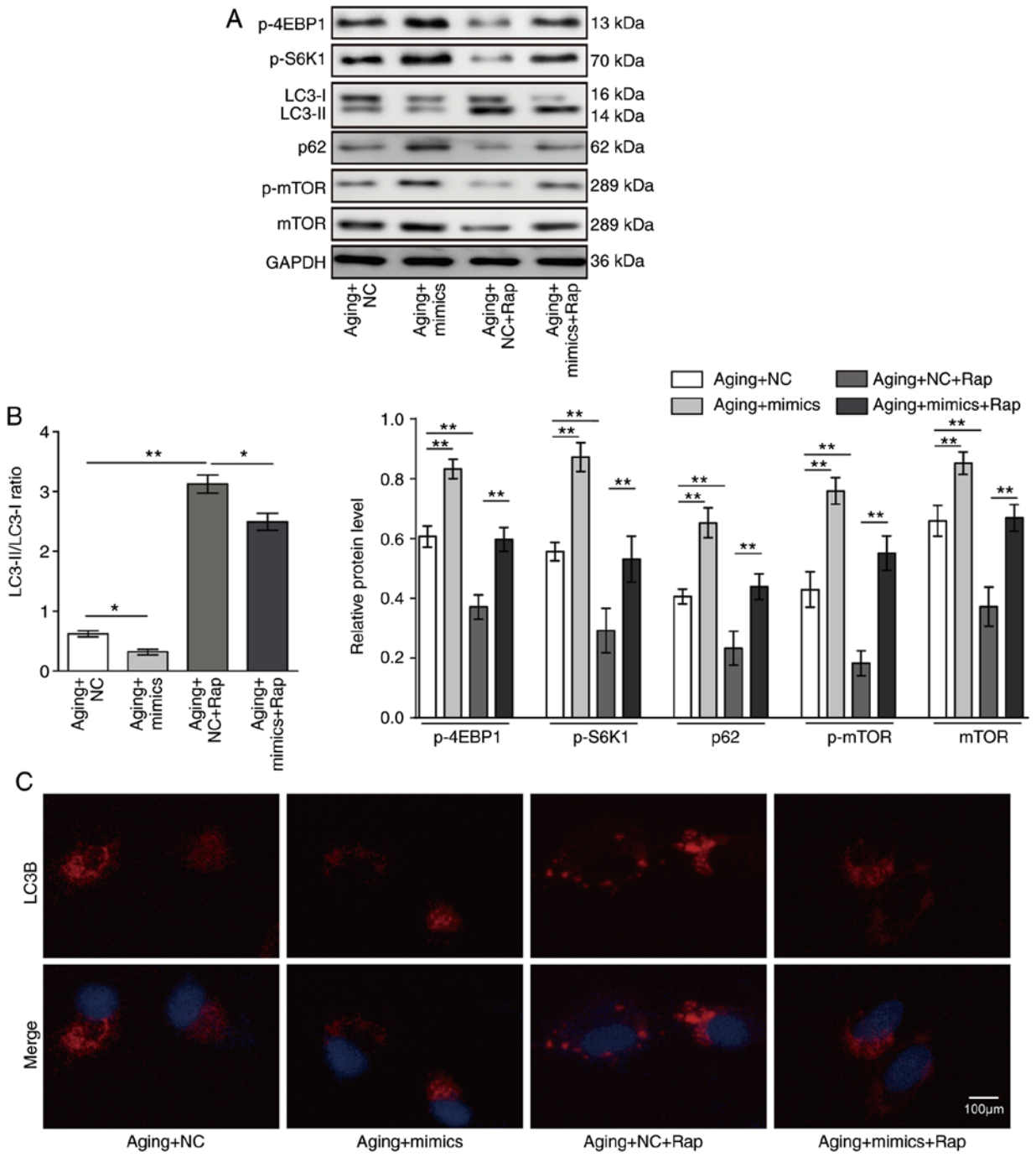

Figure 5. Rapamycin promotes autophagy in VSMCs by inhibiting miR-30a. VSMCs were transfected with miR-30a mimics or negative control for 48 h, and then treated with 20 nM rapamycin for 12 h. (A) Protein expression levels of LC3, Beclin1, p62, mTOR, p-mTOR, p-S6K1 and p-4EBP1 were determined by western blotting. Representative blots are shown. (B) Quantitative analysis of indicated proteins. (C) Immunofluorescence analysis for LC3B. Results are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by lines. VSMCs, vascular smooth muscle cells; LC3, microtubule-associated protein 1 light chain 3 β; mTOR, mammalian target of rapamycin; p-, phosphorylated; S6K1, ribosomal protein S6 kinase B1; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; NC, negative control; Rap, rapamycin.

kinase 1 (ULK1), an upstream component of autophagy initiation, is phosphorylated and inhibited by mTOR (11). Furthermore, ULK1 induces autophagy by phosphorylating Beclin1 (30) and the guanine nucleotide exchange factor DENN domain containing 3 (DENND3) (31). A previous study demonstrated that rapamycin inhibits ULK phosphorylation and induces autophagy (32). This might imply that rapamycin may promote ULK-mediated Beclin1 and DENND3 phosphorylation by inhibiting the effect of mTOR on ULK expression, thereby resulting in autophagy. In the present study, it was demonstrated that in both young and aging VSMCs, rapamycin induced autophagy by increasing the LC3-II/LC3-I ratio and the expression of Beclin1 and p-Beclin1, as well as inhibiting expression of miR-30a and mTOR. Althought rapamycin is a well-known mTOR inhibitor, the effect of rapamycin on mTOR expression levels is different in different cell lines. It has been reported that rapamycin has no effect on the expression of mTOR in the human cells Hela,

HEK293T and DU145 (33), however other studies have demonstrated that rapamycin decreases mTOR levels in the human U87-MG cell line (34) and mouse bone marrow mesenchymal stem cells (35). This difference may be due to the different cell lines, and requires further confirmation studies. In the present study, rapamycin was demonstrated to decrease mTOR levels in VSMCs. Additionally, miR-30a mimics have been reported to blunt activation of rapamycin-induced autophagy induced in cancer cells (19). Another study demonstrated that rapamycin could partly decrease the expression of miR-30a (20). Since miR-30a has been proven to be a potent suppressor of autophagy (36,37), it can be speculated that the promotion effect of rapamycin on autophagy might be partly due to the downregulation of miR-30a.

miR-30a is considered an aging-related miRNA (17). Studies have also showed that miR-30a induces cell cycle arrest. Overexpression of miR-30a increases cell apoptosis and induces cell cycle arrest in non-small cell lung cancer (38). miR-30a has also been demonstrated to promote cell cycle arrest at the G1 phase (39). The present study revealed that miR-30a induced G1 cycle arrest and promoted cellular senescence in aging VSMCs, while rapamycin significantly reversed the miR-30a-mediated cycle arrest and senescence effects through inhibition of p53/p21 signaling. These results suggested that rapamycin alleviated cycle arrest of VSMCs via downregulating miR-30a, which then further inhibited senescence of VSMCs.

Beclin1 is a factor closely related with autophagy, and it is significantly upregulated when autophagy occurs. Studies have already proven that Beclin1 is a target of miR-30a (19). miR-30a has also been demonstrated to suppress Beclin1-mediated autophagy and further sensitized tumor cells to cis-platinum (40). A previous study reported that downregulation of miR-30a could release cerebral chemic injury through enhancing Beclin1-mediated autophagy (41). The present study further demonstrated that overexpression of miR-30a could inhibit the autophagy of VSMCs by regulating expression of Beclin1, p62, p-mTOR and mTOR, by directly binding the 3'-UTR of Beclin1. These results strongly indicate that rapamycin might induce autophagy by inducing expression of Beclin1 through inhibition of miR-30a in a vascular model. Although a previous study has suggested the relationship among miR-30a, Beclin1 and rapamycin in cancer cells (19), this had not been elucidated in vascular cells and may be different depending on cell type. To the best of our knowledge, the effects of rapamycin and miR-30a on senescence have not been studied before in a vascular model. The present study demonstrated for the first time that rapamycin-induced miR-30a downregulation could alleviate VSMC senescence by regulating autophagy.

In conclusion, the current study firstly demonstrated that rapamycin inhibited the senescence of VSMCs by downregulating miR-30a, which resulted in upregulation of Beclin1 and activation of autophagy. These results clearly demonstrated that miR-30a might be a novel target for the induction of autophagy by rapamycin, and rapamycin might be a potential treatment method for senescence-related diseases.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

PT and YL designed the experiments. PT, HW, JZ, XM and XC performed the experiments. PT, YJW, YW and JZ analyzed the data. PT and YL wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols involving animals were approved by the Ethics Committee of the Department of Laboratory Animal Science, Central South University (Changsha, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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