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Inhibiting lncRNA ROR suppresses growth, migration and angiogenesis in microvascular endothelial cells by up-regulating miR-26

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Abstract. – OBJECTIVE: Lower extremity arteriosclerosis is one of leading causes of death worldwide. Arteriosclerosis is closely related to microvascular endothelial cells. This study was aimed to explore the role of long non-coding RNA ROR in regulations of growth, migration, and angiogenesis of microvascular endothelial cells.

MATERIALS AND METHODS: Angiogenesis was determined by the number of tube-like cells on a matrigel extracellular matrix. Cell viability, apoptosis, and migration were determined by CCK-8 assay, PI/FITC-Annexin V staining method, and transwell assay, respectively. Relative RNA expression of ROR, miR-26, and angiogenesis-associated genes were analyzed by qRT-PCR. The protein expression of apoptosis- and angiogenesis-associated genes, as well as main factors in NF-κB and JAK1/STAT3 pathways, were analyzed by Western blot.

RESULTS: LncRNA ROR silence inhibited viability, migration, and angiogenesis of HMEC-1 cells but promoted apoptosis of them. miR-26 expression was promoted after knocking down ROR expression. miR-26 overexpression enhanced the inhibitory effects of ROR silence on growth, migration, and angiogenesis in HMEC-1 cells, whereas, miR-26 silence impaired the effects of ROR silence. Finally, we found that NF-κB and JAK1/STAT3 signaling pathways were inhibited by ROR down-regulation. Similarly, miR-26 overexpression enhanced the inhibitory effect of ROR down-regulation on the pathways and miR-26 inhibition abrogated it.

CONCLUSIONS: Down-regulating lncRNA ROR inhibited growth, migration and angiogenesis of microvascular endothelial cells possibly through up-regulation of miR-26. During this process, the activations of NF-κB and JAK1/STAT3 pathways were inhibited after interaction of ROR and miR-26.

Key Words:
LncRNA ROR, miR-26, Angiogenesis, Arteriosclerosis, NF-κB pathway, JAK1/STAT3 pathway.

Abbreviations

LEA, lower extremity arteriosclerosis; HMEC, human microvascular endothelial cell; LncRNAs, long noncoding RNAs; miRNAs, microRNAs; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; CCK-8, Cell Counting Kit-8; PI, propidium iodide; FITC, fluorescein isothiocynate; SDS sodium dodecyl sulfate; SD, standard deviation; ANOVA, analysis of variance; NC, negative control; sh-RNA, short-hairpin RNA; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; eNOS, endothelial nitric oxide synthase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells..

Introduction

Lower extremity arteriosclerosis (LEA) was characterized by the formation of atherosclerotic plaques, which induced stenosis occlusion of lower extremity artery and further resulted in chronic limb ischemia. With the constant improvement of people's living standard, the change of life style, and the ageing of the population, the morbidity of LEA is rising year by year. It is well known that the main pathological characteristic of atherosclerosis is plaque buildup and neointima formation within large and medium-sized artery. It was reported[1,2] that angiogenesis played

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an important role in creating vulnerable sites for atherosclerotic plaques. The formation of a large number of new blood vessels in the plaques, resulting in hemorrhage and thrombosis, is closely related to the emergence of LEA disease[3]. Therefore, developing novel anti-angiogenic factors appears emergent for anti-atherosclerosis therapy. Long non-coding RNAs (LncRNAs), by definition, were transcripts longer than 200 nucleotides not translated into protein. MicroRNAs (miRNAs) are small non-coding RNA molecules (containing about 22 nucleotides) functioning in RNA silencing as well as post-transcriptional regulation of gene expression. Many lncRNAs and miRNAs were showed to participate in pathogenesis and progression of arteriosclerosis, and miRNAs were specifically mediated by lncRNAs, like TUG1-miR-26a axis[4], RNCR3-miR-185-5p axis[5], and LOC100129973-miR-4707-5p/miR-4767 axis[6]. LncRNA ROR (also called large intergenic non-coding RNA (lincRNA)-ST8SIA3), located at 18q21.31 in chromatin, was first found in induced pluripotent stem cells[7]. Pluripotency related ROR acts as a critical miRNA sponge to link miRNAs and some core transcription factors, including Oct4, Sox2 and Nanog, in human embryonic stem cells for regulating their maintenance and differentiation[8]. Increasing evidence[9] investigated the role of ROR and the possible mechanism in human cancers. For example, ROR contributed to tumorigenesis and metastasis of breast cancer and induced epithelial-to-mesenchymal transition[10]. Besides, it promoted proliferation, migration, and invasion of gallbladder tumor cells and also played an oncogenic role in non-small-cell lung cancer (NSCLC)[11,12]. However, whether ROR plays functional roles in the arteriosclerosis has not been clear. Herein, this study explored the effects of lncRNA ROR on viability, migration, and apoptosis of microvascular endothelial cell line HMEC-1. Importantly, we also detected the effect of ROR on angiogenesis. It was reported that vascular differentiation and apoptosis were mediated by transforming growth factor-β (TGF-β), which was a known target of miR-26[13]. Thus, the role of miR-26 in the functions of ROR was investigated in this study.

Materials and Methods

Cell Culture and Transfection

Human microvascular endothelial cell line, HMEC-1, purchased from the ATCC (Manassas, VA, USA), was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator. Short-hairpin RNA against human ROR (sh-ROR) and a non-targeting sequence (used as a negative control (NC)) were designed and transfected into cells with Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA). The synthesized miR-26 mimic, inhibitor and their respective NC (Life Technologies Corporation, Carlsbad, CA, USA) were transfected into cells. After collecting transfected cells after 48 h, the transfection efficiency was determined.

qRT-PCR Analysis

RNA was extracted through TRIzol reagent (Beyotime, Shanghai, China). The relative expression levels of ROR, miR-26, and angiogenesis-related genes were analyzed by qRT-PCR. The One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa, Otsu, Shiga, Japan) was used for analyses of ROR and angiogenesis-related genes. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-26 and U6 (Applied Biosystems, Foster City, CA, USA) were used for analyses of miR-26. GADPH and U6 were used as the respective endogenous control for ROR and angiogenesis-related genes and miR-26. Their relative expression was calculated using ($2^{-\Delta\Delta Ct}$) method.

CCK-8 Assay

HMEC-1 cells were seeded in the 96-well plate with a density of 5×10^3 cells/well. After different treatments, CCK-8 solution purchased from Beyotime (Shanghai, China) was added in the wells. After incubation for 1 h at 37°C in an incubator with 5% CO_2, absorbance was obtained using a Microplate Reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Apoptosis Assay

Cells after different treatment were incubated with PI/FITC-Annexin V solution containing 50 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature in the dark; next, they were washed with PBS. Apoptosis of cells was analyzed by flow cytometry using a FACSCalibur (Beckman Coulter, Brea, CA, USA) and the data were analyzed using FlowJo software.

Migration Assay

The transwell chamber with pore size of 8 μm (Costar, Cambridge, MA, USA) was used to determine migration. Cells were suspended in 200 μl of serum-free medium in the upper chamber, and 600 μl of medium containing serum were added in the lower chamber. After incubation at 37°C for 24 h, non-migrated cells were removed using swab; and migrated cells were fixed in methanol for 30 min and stained with crystal violet (Beyotime, Shanghai, China) for 20 min. Finally, migrated cells were counted by microscope.

Western Blot

Proteins were isolated from HMEC-1 cells by incubation with RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Obtained protein was quantified by BCA™ Protein Assay Kit (Beyotime, Shanghai, China). SDS gel electrophoresis was performed to separate proteins on NuPAGE bistris gradient gels (4%-12%) using MES running buffer (Beyotime, Shanghai, China). Proteins were then transferred onto nitrocellulose membranes. After blocking in 5% bovine milk for 1 h, nitrocellulose membranes carrying proteins were probed with the following primary antibodies: VEGF (#ab1316), VEGFR2 (#ab11939), CD144 (#ab166715), eNOS (#ab76198), pro-Caspase-3 (#ab2302), cleaved-Caspase-3 (#ab2302), pro-Caspase-9 (#ab32539), cleaved-Caspase-9 (#ab32539), Bcl-2 (#ab32124), Bax (#ab32503), p65 (#ab16502), p-p65 (#ab86299), IκBα (#ab32518), p-IκBα (#ab133462), JAK1 (#ab47435), p-JAK1 (#ab138005), STAT3 (#ab68153), p-STAT3 (#ab76315), and β-actin (#ab8226, Abcam, Cambridge, MA, USA). After incubation at 4°C overnight, the membranes were probed with the appropriate secondary antibody for 1 h at room temperature. The signals were captured by autoradiography using ECL reagents (GE Healthcare Life Sciences, UK), and the densitometry was quantified through Image J software.

In Vitro Angiogenesis (Tubulogenesis) Assay

HMEC-1 cells seeded in a matrigel (BD Biosciences, Franklin Lakes, NJ, USA) extracellular matrix-coated 6 well plate with density of 4×10^4 cells per well. After 24 h of culture, cells were subjected to phase-contrast microscopy. Tubulogenesis was determined by the number of tube-like cells [14].

Statistical Analysis

All statistical analyses were performed by Graph Pad Prism version 6.0 software (Graph Pad Software, La Jolla, CA, USA) using one-way or two-way ANOVA followed by Tukey post-hoc test. The data were expressed as means + SD from three independent experiments. $p < 0.05$ indicated statistically significant results.

Results

Angiogenesis in HMEC-1 Cells

The tube-like structures of human vascular endothelial cell line HMEC-1 were found when cells were seeded onto extracellular matrix. The ratio of number of tube-like cells to nucleus was gradually increased with increasing incubation time (Figure 1A). Afterwards, angiogenesis-associated genes were detected by qRT-PCR and Western blot. The mRNA levels of VEGF, VEGFR2, CD144, and eNOS were significantly enhanced after incubation at 6 h ($p < 0.01$, $p < 0.01$, $p < 0.001$, and $p < 0.01$), 12 h (all $p < 0.001$) and 24 h (all $p < 0.001$, Figure 1B). The similar changes occurred on their protein expression (Figure 1C). We found that VEGF and eNOS expression were significantly increased at 6 h, 12 h, and 24 h (all $p < 0.001$), VEGFR2 expression was significantly elevated at 6 h, 12 h, and 24 h ($p < 0.05$, $p < 0.001$, and $p < 0.001$), and CD144 expression was significantly increased at 1 h, 6 h, 12 h, and 24 h ($p < 0.05$, $p < 0.001$, $p < 0.001$, and $p < 0.001$).

Down-Regulating ROR Inhibited Growth, Migration, and Angiogenesis in HMEC-1 Cells

The effects of ROR on viability, migration, angiogenesis, and apoptosis of HMEC-1 cells were analyzed. ROR expression was silenced after transfection with sh-ROR#1 and 2 ($p < 0.05$ and $p < 0.01$, Figure 2A). sh-ROR#2 was used to knock down ROR in HMEC-1 cells due to its better transfection effect. The effects of sh-ROR#2 treatment on HMEC-1 cells were investigated. Transfection with sh-ROR#2 significantly decreased viability ($p < 0.05$, Figure 2B), migration ($p < 0.05$, Figure 2C), and tubes/nucleus ratio ($p < 0.05$, Figure 2D), but increased apoptosis ($p < 0.01$, Figure 2E). We also found that Caspase-3/9 was activated, Bcl-2 expression was decreased, and Bax expression was increased after knocking down ROR (Fig-

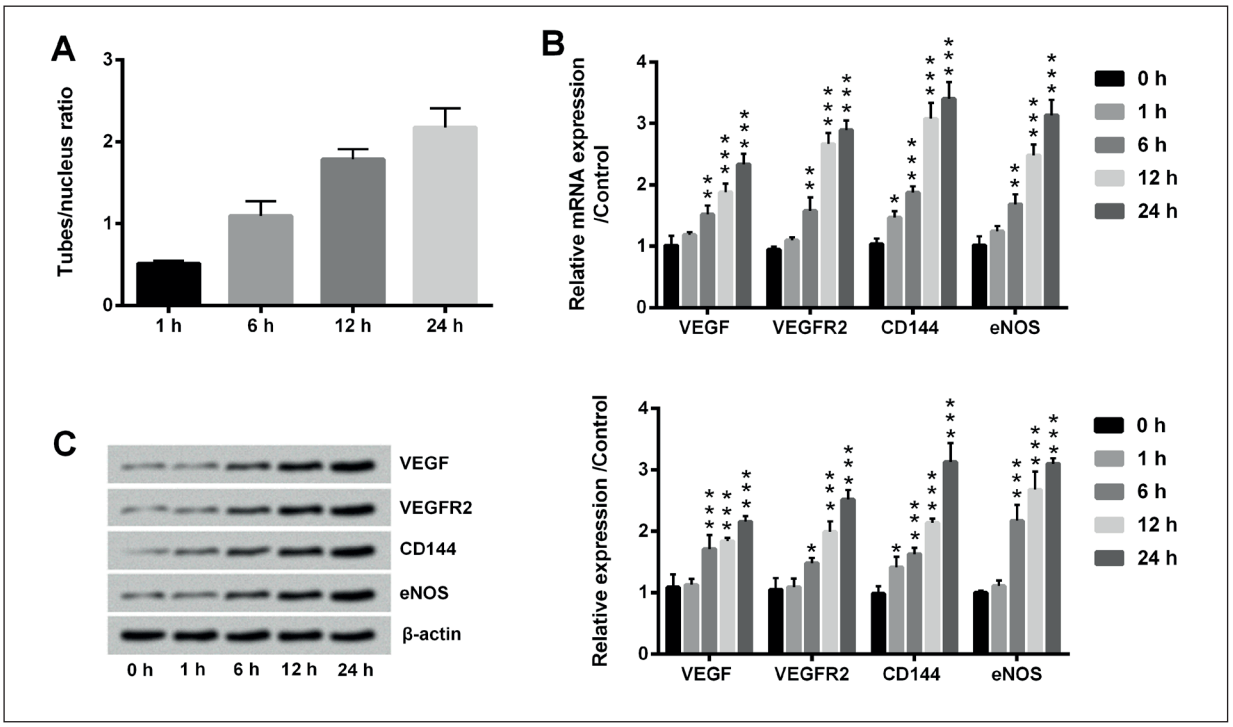

Figure 1. Angiogenesis of HMEC-1 cells was increased with prolonging incubation time. The degrees of angiogenesis were evaluated by ***(A)*** calculating formed tubes/nucleus ration, ***(B)*** detecting the mRNA expression of angiogenesis-associated genes, and ***(C)*** detecting the protein expression of angiogenesis-associated genes.

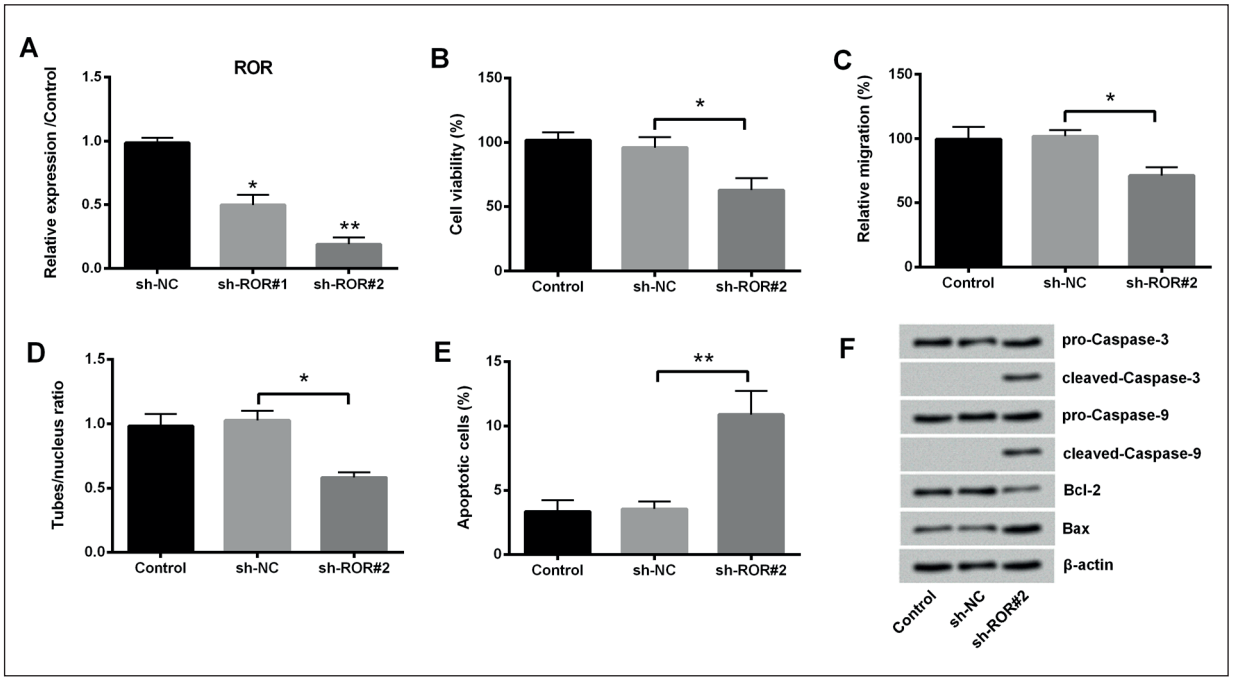

Figure 2. ROR silence inhibited growth, migration, and angiogenesis in HMEC-1 cells. ***(A)*** ROR expression was silenced after transfection with sh-ROR#1 and 2. ***(B)*** Cell viability, ***(C)*** migration, and ***(D)*** angiogenesis were inhibited in sh-ROR#2-transfected cells. ***(E)*** Apoptotic cell rate of sh-ROR#2-transfected cells was enhanced and ***(F)*** the expression of proteins linked with apoptosis were altered by sh-ROR#2 treatment.

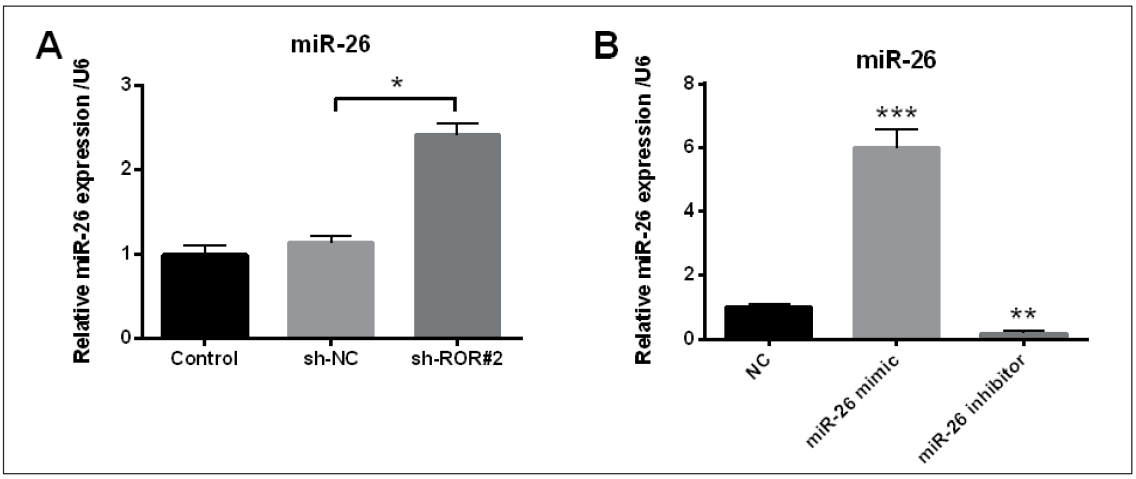

Figure 3. ROR silence up-regulated miR-26 expression. ***(A)*** miR-26 expression was negatively regulated by ROR. ***(B)*** miR-26 was overexpressed and knocked down in miR-26 mimic- and miR-26 inhibitor-transfected cells.

ure 2F). Results indicate that inhibiting ROR expression in HMEC-1 cells suppressed their growth, migration, and angiogenesis.

miR-26 Enhanced the Inhibitory Effects of ROR Silence on Growth, Migration, and Angiogenesis in HMEC-1 Cells

A significant increase of miR-26 expression was found in sh-ROR#2-transfected HMEC-1 cells ($p < 0.05$, Figure 3A). The role of miR-26 in the effects of ROR on HMEC-1 cells was investigated due to the regulation of ROR on miR-26. miR-26 expression was significantly increased and decreased after transfection with miR-26 mimic and inhibitor, respectively ($p < 0.001$ and $p < 0.01$, Figure 3B). After that, the effects of miR-26 mimic and inhibitor on viability, migration, angiogenesis, and apoptosis of ROR-silenced cells were analyzed. Results showed that miR-26 mimic treatment further enhanced the inhibiting effects of ROR on viability ($p < 0.05$, Figure 4A), migration ($p < 0.05$, Figure 4B), and angiogenesis ($p < 0.05$, Figure 4C), as well as apoptosis-promoting effect ($p < 0.05$, Figure 4D). However, miR-26 inhibitor treatment showed the remarkable opposite effects on viability ($p < 0.05$, Figure 4A), migration ($p < 0.05$, Figure 4B), angiogenesis ($p < 0.05$, Figure 4C), and apoptosis

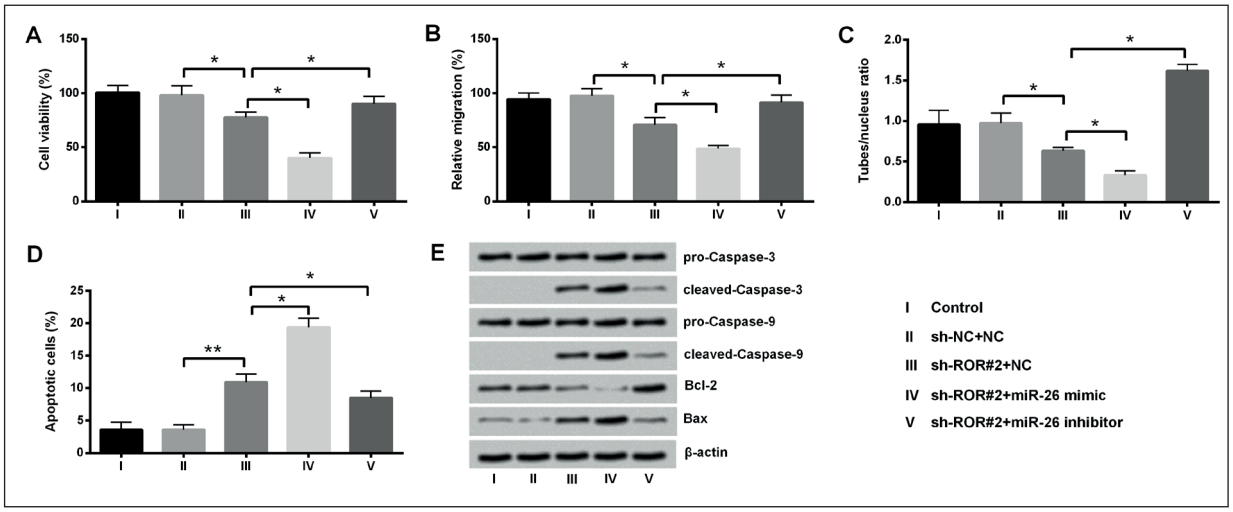

Figure 4. ROR silence inhibited growth, migration, and angiogenesis of HMEC-1 cells by up-regulating miR-26. miR-26 overexpression enhanced the inhibitory effects of ROR suppression on ***(A)*** cell viability, ***(B)*** migration, and ***(C)*** angiogenesis, and ***(D)*** also reduced the promoting effect of ROR on apoptosis. ***(E)*** miR-26 modulated the effects of ROR on apoptosis-related proteins.

($p < 0.05$, Figure 4D). miR-26 overexpression and silence exhibited the contrary effects on cleaved-Caspase-3, cleaved-Caspase-9, Bax, and Bcl-2 expression in ROR-silenced cells (Figure 4E). All data suggest that miR-26 overexpression might contribute to the growth-, migration-, and angiogenesis-inhibitory effects of ROR suppression.

miR-26 Enhanced the Inhibitory Effects of ROR Silence on NF-κB and JAK1/STAT3 Pathways in HMEC-1 Cells

Signaling pathways involved in the function of ROR were studied to clarify the possible action mechanism of ROR-miR-26 axis. According to our data, levels of phosphorylated p65 and IκBα were decreased after inhibiting ROR expression, then further declined after inhibiting ROR and simultaneously elevating miR-26 expression, but they were increased after inhibiting ROR and miR-26 expression (Figure 5A). The similar changes were found on expression of phosphorylated JAK1 and STAT3 (Figure 5B). These results indicate that knocking down ROR inhibited NF-κB and JAK1/STAT3 pathways in HMEC-1 cells possibly by up-regulating miR-26, which might resulted in inhibitions of cell viability, migration, and angiogenesis.

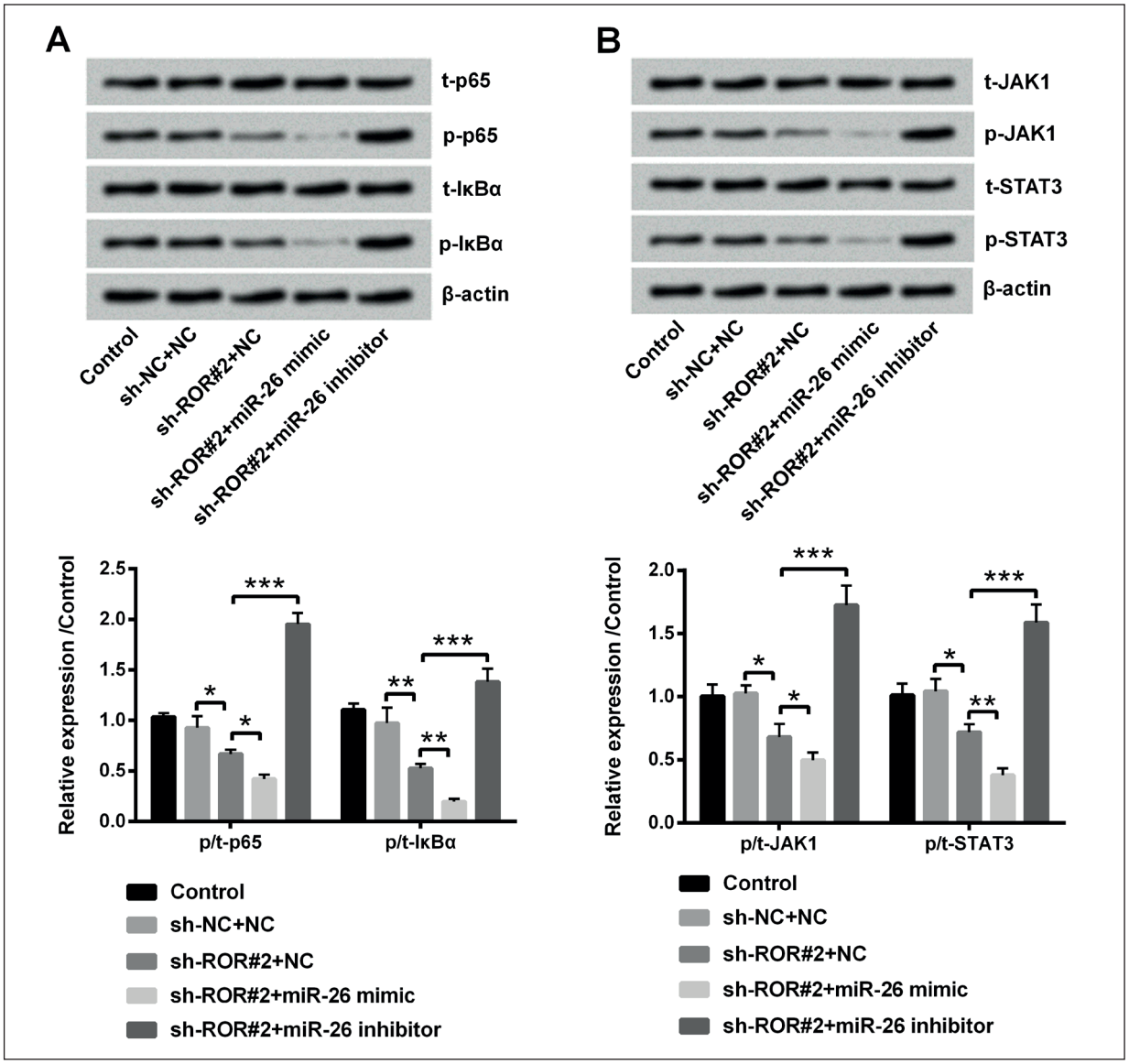

Figure 5. ROR silence suppressed activations of NF-κB and JAK1/STAT3 pathways by up-regulating miR-26. ***(A)*** miR-26 overexpression increased the inhibitory effect of ROR on NF-κB pathway and miR-26 silence exhibited the contrary effect. ***(B)*** miR-26 overexpression enhanced the inhibitory effect of ROR on JAK1/STAT3 pathway and miR-26 exerted the contrary effect.

Discussion

Atherosclerosis is a common chronic inflammatory disease, which is the major reason of myocardial infarction and stroke in middle-aged and elderly people[15]. Therefore, suppressing atherosclerosis is important for preventing some life-threatening complications[16]. It is well-known that abnormal viability, proliferation, metastasis, and angiogenesis of endothelial cells induce alteration in endothelial function, which plays vital roles in the origination and progression of atherosclerotic lesions[17]. We used human microvascular endothelial cell line (HMEC-1 cell) to investigate the effects of lncRNA ROR on growth, migration, and angiogenesis of them for evaluating the potential function of ROR in inhibition of plaque formation and treatment of atherosclerosis. The tube-like cell number was counted by *in vitro* angiogenesis assays, and all quantifications were corrected to the total nuclei number for avoiding any cell number bias, and data were presented as tubes/nuclei ratio[14]. Besides, well-recognized angiogenic factors, VEGF, VEGFR2, CD144, and eNOS expression were detected to evaluate the degree of angiogenesis. We found that high tube/nucleus ratio was observed after culture and expression of VEGF, VEGFR2, CD144, and eNOS expression were also enhanced. However, the tube/nucleus ratio was decreased after LncRNA ROR was silenced in HMEC-1 cells. This data directly indicates the anti-angiogenic effect of ROR silence. Meanwhile, viability and migration were inhibited and apoptosis was promoted in ROR-silenced cells. Endothelial cell proliferation and migration are two crucial steps of angiogenesis [2]. Switching to the angiogenic phenotype of endothelial cells might involve suppression of apoptosis[18]. Thus, we detected the effects of ROR on viability, migration, and apoptosis of HMEC-1 cells and the results also demonstrated that down-regulating ROR inhibited the switch of angiogenic phenotype of HMEC-1 cells. The endothelium has been considered as a key regulator of vascular homeostasis because of possessing barrier functions and acting as an active signal transducer for the circulating influence, which modifies the vessel wall phenotype[19,20]. A subset of lncRNAs and miRNAs have been identified as important coordinators of atherosclerosis; however, researches on their participation in endothelial activities in atherosclerosis have been limited[16,21]. This is the first study investigating the roles of lncRNA ROR in *in vitro* angiogenesis assay and in endothelial development. lncRNA ROR was reported to interact with miR-145 promoting the self-renewal of embryonic stem cells[8] and interact with miR-133 promoting cardiac hypertrophy[7]. The underlying action mechanism of ROR down-regulation in HMEC-1 cells was explored. Researches showed the vital role of miR-26 in cardiovascular disease. Sun et al[22] reported the function of miR-26 in the regulation of cholesterol metabolism via targeting ABCA1 and ARL7. It was reported that the evolution from fatty streaks to fibrous atheroma was facilitated by proliferation of vascular smooth muscle cells in the neointima and vascular differentiation and apoptosis were mediated by TGF-β, a vital target of miR-26a[13]. miR-26a was identified to inhibit angiogenesis in endothelial cells by targeting the SMAD1–Id1–$p21^{WAF/CIP1}$/p27 signaling axis, whereas inhibition of it induced angiogenesis[23]. miR-26a also blocked exercise-induced angiogenesis in skeletal muscle by targeting BMP/SMAD1[24]. Overall, these findings provide cogent evidence that miR-26 serves an anti-angiogenic role in HMEC-1 cells. Multiple genes whose products participate in process of atherosclerosis are regulated by NF-κB signaling[15,25]. NF-κB signaling was showed to orchestrate expression of pro-inflammatory genes at the arterial wall and promote the atherosclerotic pathogenesis[26]. Endothelial cell-specific NF-κB inhibition could protect mice from atherosclerosis[26]. The vascular endothelial activation was reported to be induced by IL-6 dependent JAK1/STAT3 signaling, which might further contribute to cardiovascular diseases[27]. JAK1/STAT3 signaling was an important target to inhibit angiogenesis in some cancer cells[28] and vein endothelial cells[29], as well as regulate regeneration and repair of spinal cord injury[30]. In this study, we found that NF-κB and JAK1/STAT3 pathways were inhibited in response to ROR down-regulation and miR-26 up-regulation.

Conclusions

We demonstrated that down-regulating lncRNA ROR inhibited growth and migration of HMEC-1 cells and more importantly inhibited the capillary tube formation. miR-26 overexpression enhanced the function of ROR silence in HMEC-1 cells. Finally, we found that suppression of ROR inhibited NF-κB and JAK1/STAT3 signaling by elevating miR-26 expression. The study might provide new insights into the targeting therapies of angiogenesis in atherosclerosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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