Recombinant chromosome 6 open reading frame 120 protein promotes angiogenesis and endothelial-to-mesenchymal transition in human umbilical vein endothelial cells via the PI3K/Akt signaling pathway

YINGYING LIN[1*], XIN WANG[1*], YANYAN LI[2], XINYU CUI[2], NA ZHU[2] and XIN LI[1]

[1]Department of Center of Integrated Traditional Chinese and Western Medicine, Peking University Ditan Teaching Hospital, Beijing 100015, P.R. China; [2]Department of Center of Integrated Traditional Chinese and Western Medicine, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, P.R. China

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Abstract. The vascular endothelium plays a pivotal role in modulating various physiological processes and its dysfunction is fundamental to the development of numerous vascular and non-vascular diseases. Chromosome 6 open reading frame 120 (C6ORF120) has been implicated in cellular processes such as apoptosis, inflammation, immunomodulation and fibrosis. However, the specific effects of C6ORF120 on endothelial cell function remain unclear. The present study aimed to explore the potential role of C6ORF120 in endothelial dysfunction and its underlying molecular mechanisms. It synthesized recombinant C6ORF120 protein (rC6ORF120) and assessed its effects on human umbilical vein endothelial cells (HUVECs) through various functional assays, including the CCK-8 assay for proliferation, scratch assay for migration and tube formation assay for angiogenesis. Additionally, immunofluorescence (IF) and western blotting (WB) were employed to evaluate endothelial-mesenchymal transition (EndMT). The present study also quantified the expression of key proteins within the PI3K/Akt signaling pathway to elucidate its role in mediating the effects of rC6ORF120 on HUVECs. Treatment with rC6ORF120 significantly enhanced HUVEC proliferation (200 ng/ml vs. control at 72 h, 1.14±0.01 vs. 1.05±0.02; t=8.15; P<0.001) and induced phenotypic changes. In migration and angiogenesis assays, rC6ORF120-treated HUVECs exhibited increased wound closure (37.69±2.74% vs. 66.16±6.13%; t=7.35; P=0.002) and angiogenesis assays showed significant improvements in tube formation parameters such as total tubule length (77,199.67±4,684.88 μm vs. 96,203.00±3,354.89 μm; t=5.71; P=0.002). WB and IF analyses both indicated that rC6ORF120 promotes EndMT in HUVECs. Furthermore, rC6ORF120 treatment increased PI3K/Akt phosphorylation significantly compared with controls (p-PI3K; 1.57±0.18 vs. 1.00±0.00; t=5.64; P=0.005). LY294002 significantly reversed these effects on EndMT and angiogenesis (P<0.05), while the effect on cell migration was less pronounced (P=0.565). Our study highlights the critical role of C6ORF120 in HUVECs, promoting proliferation, migration, angiogenesis and EndMT, which are mediated, at least in part, by the PI3K/Akt pathway.

Introduction

The human endothelial layer, composed of endothelial cells, is one of the largest organ systems in the human body, playing a critical role in maintaining physiological homeostasis through immune modulation, signal transduction, redox equilibrium and regulation of vascular tone (1). When endothelial integrity is compromised, endothelial cells undergo a series of pathological changes termed endothelial reprogramming, primarily characterized by endothelial-to-mesenchymal transition (EndMT) and pathological angiogenesis (2,3). During endothelial reprogramming, endothelial cells transition from a quiescent phenotype to an activated state, accompanied by enhanced migration and angiogenic capacities (3,4). Such endothelial dysfunction markedly contributes to the pathogenesis of various diseases, including inflammation, atherosclerosis, thrombosis, hypertension and fibrosis (5).

EndMT was first identified during endocardial differentiation (4) and has since been recognized as a pivotal mechanism involved in developmental anomalies, tumor progression and various fibrotic diseases (4,6,7). For instance, in liver fibrosis, EndMT is considered an important source of fibroblasts and

Correspondence to: Professor Xin Li, Department of Center of Integrated Traditional Chinese and Western Medicine, Peking University Ditan Teaching Hospital, 8 Jingshun East Street, Chaoyang, Beijing 100015, P.R. China
E-mail: leaxin@ccmu.edu.cn

*Contributed equally

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contributes to hepatic stellate cell activation (8). Additionally, EndMT is closely associated with disease progression, pathological angiogenesis and drug resistance in conditions such as atherosclerosis and cancer (6,7). During the EndMT process, cells acquire an intermediate phenotype characterized by markedly enhanced angiogenic, migratory and proliferative capabilities (9,10). Thus, elucidating the mechanisms underlying endothelial dysfunction and reprogramming could provide valuable insights into the pathogenesis of these diseases and potentially identify novel therapeutic targets.

Chromosome 6 open reading frame 120 (C6ORF120) is a recently identified secreted protein implicated in multiple physiological and pathological processes. Previous studies have demonstrated its association with various diseases, including AIDS (11), diabetes (12), autoimmune hepatitis (13-15) and liver fibrosis (16). Our prior studies revealed that C6ORF120 could regulate physiological and pathological processes such as apoptosis, immune response and fibrogenesis through its effects on hepatocytes (17), CD4 T lymphocytes (15) and hepatic stellate cells (16). However, the role of C6ORF120 in endothelial cells has not yet been explored. The present study aimed to investigate its potential involvement in endothelial cell biology, as understanding the function of C6ORF120 in endothelial cells could provide new insights into not only liver diseases but also vascular and fibrosis-related conditions beyond the liver. It was hypothesized that C6ORF120 may play a previously unexplored role in endothelial dysfunction, which could have implications for a wide range of vascular and fibrotic diseases.

Human umbilical vein endothelial cells (HUVECs), due to their representative and accessible nature, are extensively utilized as a classical endothelial cell model in vascular biology research (18,19). Therefore, the present study aimed to investigate the effects of C6ORF120 on endothelial cell functions (particularly EndMT, angiogenesis and migration) in HUVECs and to further elucidate the underlying molecular mechanisms, thereby providing insights into the role of C6ORF120 in endothelial dysfunction.

Materials and methods

Cell culture. The HUVECs used in the present study were purchased from American Type Culture Collection (cat. no. CRL-1730) through the Institute of Infectious Diseases, Ditan Hospital, Beijing, China (20). These cells were obtained at passage 2 and used between passages 2 and 5 in all experiments (21). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; cat. no. A4192101; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 1% penicillin/streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (FBS; cat. no. A5670701; Gibco; Thermo Fisher Scientific, Inc.) and incubated in a humidified atmosphere containing 5% CO_2 at 37˚C. HUVECs were passaged when reaching 80% confluence.

Cell treatments. HUVECs in the logarithmic growth phase were seeded in 6-well plates and serum-starved in serum-free DMEM for 24 h before treatment. Cells were subsequently treated with 2 ml of medium containing recombinant C6ORF120 protein (rC6ORF120; Cusabio Technology, LLC) at indicated concentrations (15,16), TGF-β1 (10 ng/ml; BioLegend, Inc.) as a positive control (22), or the PI3K inhibitor LY294002 (10 μmol; MedChemExpress) for mechanistic studies (23,24). The control group was cultured with normal medium Each experiment was independently repeated at least three times and cells were harvested at predetermined time points for subsequent analysis.

CCK-8 assay. HUVECs (5,000 cells/well) were seeded into 96-well plates and treated with rC6ORF120 at concentrations of 0, 100, 200 and 400 ng/ml in a volume of 100 μl per well. After incubation for 24, 48 and 72 h, cell viability was evaluated using a Cell Counting Kit-8 (CCK-8; Shanghai Topscience Co., Ltd.) according to the manufacturer's instructions (25). Specifically, 10 μl of CCK-8 solution was added to each well, followed by incubation at 37˚C for 2 h. Absorbance was measured at 450 nm using a microplate reader. Each condition was tested in triplicate and the experiment was independently repeated three times.

Cell morphology assay. HUVECs were seeded in 6-well plates at a density of $2x10^5$ cells per well and treated with rC6ORF120 at concentrations of 0, 100, 200 and 400 ng/ml. Morphological changes were observed under an inverted phase-contrast microscope after 48 h of treatment and after continuous stimulation for five passages (26). Changes in cell shape (such as elongation, loss of cobblestone appearance and spindle-like transformation) were recorded. Representative images were captured from three randomly selected fields in each well and the experiments were independently repeated three times.

Western blotting. Cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) supplemented with phosphatase inhibitors and PMSF on ice for 1 h, followed by centrifugation at 12,000 x g for 15 min at 4˚C. Protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore; Merck KGaA). After blocking with 5% skimmed milk for 1 h at room temperature, membranes were incubated overnight at 4˚C with primary antibodies against CD31 (cat. no. 28083-1-AP; 1:1,000; Proteintech Group, Inc.), VE-cadherin (cat. no. 66804-1-IG; 1:1,000; Proteintech Group, Inc.), Vimentin (cat. no. 10366-1-AP; 1:5,000; Proteintech Group, Inc.), α-smooth muscle actin (α-SMA; cat. no. 67735-1-IG; 1:5,000; Proteintech Group, Inc.), AKT (cat. no. 60203-2-Ig; 1:1,000; Proteintech Group, Inc.), phosphorylated (p-)AKT (cat. no. 66444-1-Ig; 1:1,000; Proteintech Group, Inc.), PI3K (cat. no. 60225-1-Ig; 1:1,000; Proteintech Group, Inc.), p-PI3K (cat. no. ab182651; 1:1,000; Abcam) and GAPDH (cat. no. 60004-1-Ig; 1:5,000; Proteintech Group, Inc.). Membranes were washed three times with tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated with HRP-conjugated secondary antibodies (anti-rabbit: cat. no. SA00001-2; 1:5,000; anti-mouse: cat. no. SA00001-1; 1:5,000; both Proteintech Group, Inc.) for 1 h at room temperature. Bands were visualized using chemiluminescence detection as previously described (27). Experiments were independently repeated three times.

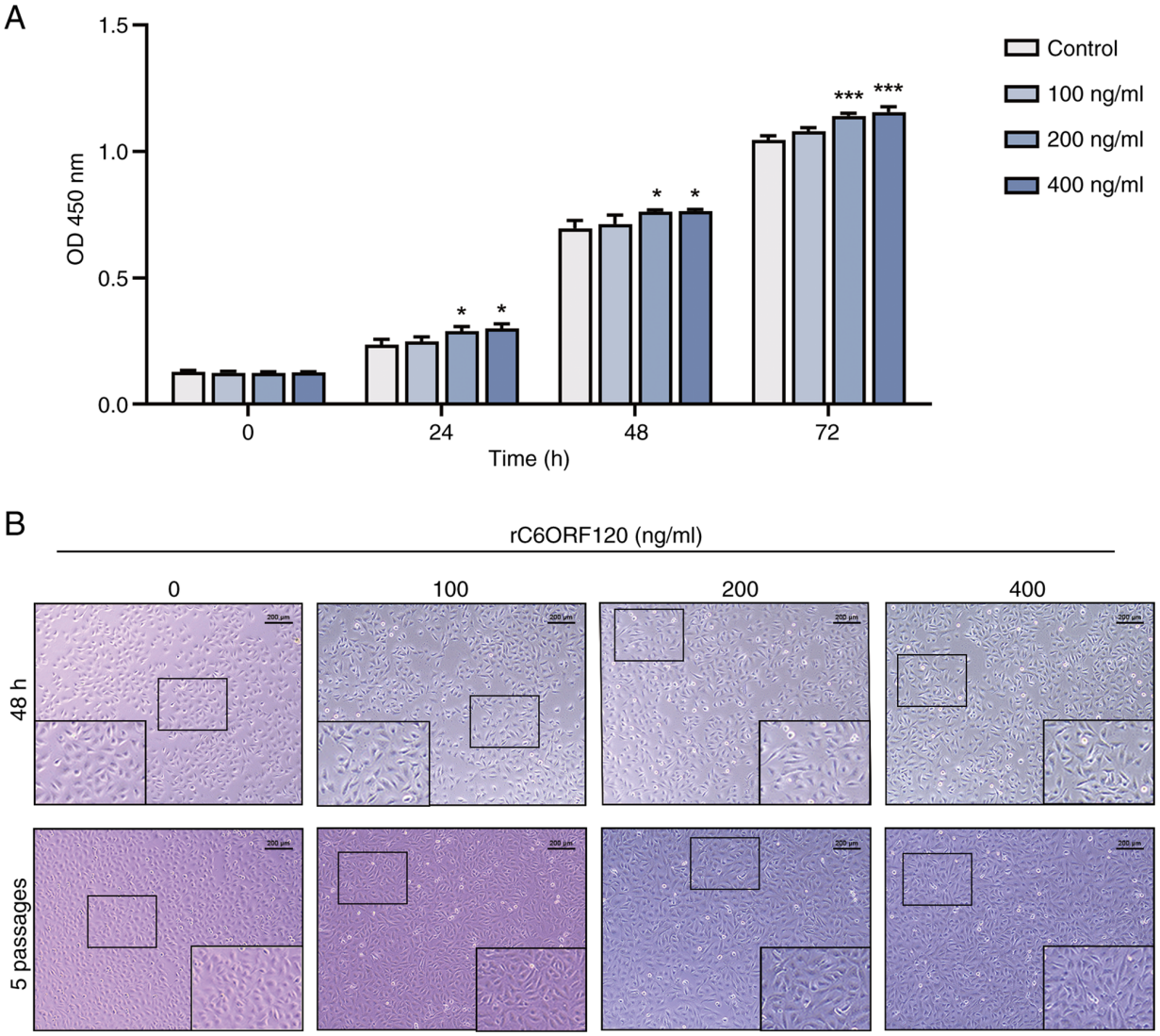

Figure 1. Effect of rC6ORF120 on HUVECs viability and morphology. (A) CCK8 assay was used to determine the viability of HUVECs at 24, 48 and 72 h after stimulation with rC6ORF120 (0, 100, 200 and 400 ng/ml). (B) Morphological alterations in HUVECs following 48-h exposure or five passages of rC6ORF120 treatment. ns indicates no statistically significant difference; *P<0.05; ***P<0.001. rC6ORF120, recombinant C6ORF120 protein; HUVECs, human umbilical vein endothelial cells.

Immunofluorescence (IF) staining assay. HUVECs were seeded on sterile glass coverslips (1x10^5 cells/ml) were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.05% Triton X-100 for 15 min and blocked with 5% bovine serum albumin (BSA) for 1 h all at 37˚C. Cells were then incubated with primary antibodies against CD31 (cat. no. 28083-1-AP; 1:500; Proteintech Group, Inc.) and α-SMA (cat. no. 14395-1-AP; 1:500; Proteintech Group, Inc.) overnight at 4˚C. After washing with PBS, cells were incubated with Alexa Fluor 555-conjugated secondary antibodies (cat. no. ab150078; 1:200; Abcam) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at room temperature (28). Fluorescence signals were visualized and imaged using a fluorescence microscope. ImageJ software (version 1.54; National Institutes of Health) was used for quantitative analysis of fluorescence intensity. Each condition was tested in triplicate and three independent experiments were conducted.

Wound healing assay. Before cell seeding, the reverse side of 6-well plates was marked with horizontal lines using a black marker to ensure consistent scratch locations. HUVECs were cultured in 6-well plates and grown to 95% confluence. A linear scratch was generated using a 10 μl pipette tip. Cells were washed gently with PBS to remove detached cells and subsequently cultured with serum-free medium corresponding to each treatment group. Wound healing was monitored at 0 and 48 h using a light microscope. Images were analyzed using ImageJ software (version 1.54; National Institutes of Health) to quantify wound closure percentage (29). Experiments were independently repeated three times.

Tube formation assay. Matrigel (Corning, Inc.) was evenly applied onto 24-well plates at 100 μl per well and incubated at 37˚C for 1 h to solidify. HUVECs suspended in medium were seeded at a density of 1.5x10^5 cells per well in a volume of 250 μl. After 8 h of incubation, tube-like structures were visualized using an inverted microscope and tube formation parameters,

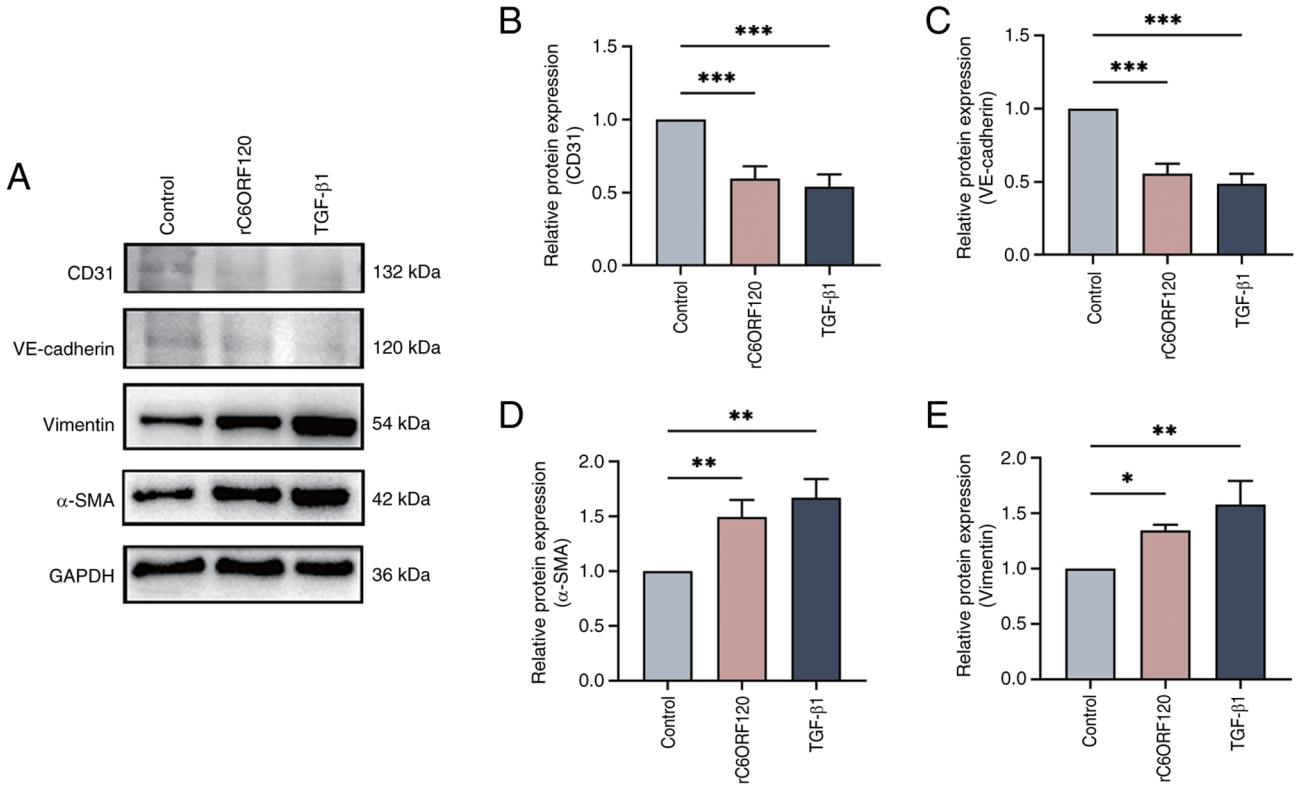

Figure 2. rC6ORF120 induced the EndMT in HUVECs. (A) Representative western blot images of endothelial (CD31 and VE-cadherin) and mesenchymal (Vimentin and α-SMA) marker proteins in HUVECs following treatment with rC6ORF120 and TGF-β1. (B-E) Corresponding bar graph representing relative expression of protein expression levels, normalized to GAPDH. *P<0.05; **P<0.01; ***P<0.001. rC6ORF120, recombinant C6ORF120 protein; EndMT, endothelial-mesenchymal transition; HUVECs, human umbilical vein endothelial cells; α-SMA, α-smooth muscle actin.

including the total vessel length and number of junctions, were quantified by ImageJ software (version 1.54) (30). Each experiment was independently repeated three times.

Statistical analyses. Data analysis was conducted using GraphPad Prism 6 software (Dotmatics). Results were expressed as mean ± standard deviation. Statistical comparisons between two groups were performed using unpaired Student's t-tests and multiple group comparisons were conducted using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

rC6ORF120 affects the viability and morphology of HUVECs. HUVEC viability significantly increased in a concentration-dependent manner after treatment with rC6ORF120 at 24, 48 and 72 h (Fig. 1A). Specifically, at 72 h, treatment with 200 ng/ml (200 ng/ml vs. control: 1.14±0.01 vs. 1.05±0.02; t=8.15; P<0.001) and 400 ng/ml (400 ng/ml vs. control: 1.16±0.02 vs. 1.05±0.02; t=6.83; P<0.001) rC6ORF120 markedly enhanced cell viability compared to the control. Due to comparable viability at 200 and 400 ng/ml (t=1.05, P=0.353), 200 ng/ml was selected for subsequent experiments.

Morphologically, control HUVECs exhibited a typical cobblestone-like morphology, whereas those exposed to rC6ORF120 underwent a time- and concentration-dependent morphological shift towards a spindle-like, fibroblast-like appearance (Fig. 1B). This transformation was more pronounced after long-term exposure compared to short-term treatment, implying a potential role of rC6ORF120 in modulating endothelial cell shape and phenotype.

rC6ORF120 induces EndMT in HUVECs. The morphological observations of HUVECs following rC6ORF120 treatment prompted the hypothesis that these cells might undergo EndMT. To confirm EndMT induction by rC6ORF120, relevant protein markers were assessed by western blotting (WB) using TGF-β1 as a positive control (4,31). Compared with controls, rC6ORF120-treated HUVECs showed significantly reduced endothelial markers CD31 (0.60±0.08 vs. control 1.00; t=8.32; P<0.001) and VE-cadherin (0.56±0.07 vs. control 1.00; t=11.39; P<0.001), along with significantly increased mesenchymal markers α-SMA (1.50±0.16 vs. control 1.00; t=5.48, P=0.005) and Vimentin (1.35±0.05 vs. control 1.00; t=11.98, P=0.007; Fig. 2A-E).

IF staining further validated these findings, indicating significantly decreased fluorescence intensity for CD31 (0.77±0.04 vs. 1.00±0.01; t=9.10; P<0.001) and increased intensity for α-SMA (1.33±0.01 vs. 1.00±0.08; t=6.57, P=0.003) in the rC6ORF120-treated group compared to control (Fig. 3). Collectively, the results indicated that rC6ORF120 induced EndMT in HUVECs, as evidenced by phenotypic changes and alterations in marker expression.

rC6ORF120 promotes migration and angiogenesis in HUVECs. Mesenchymal cells are recognized for their higher

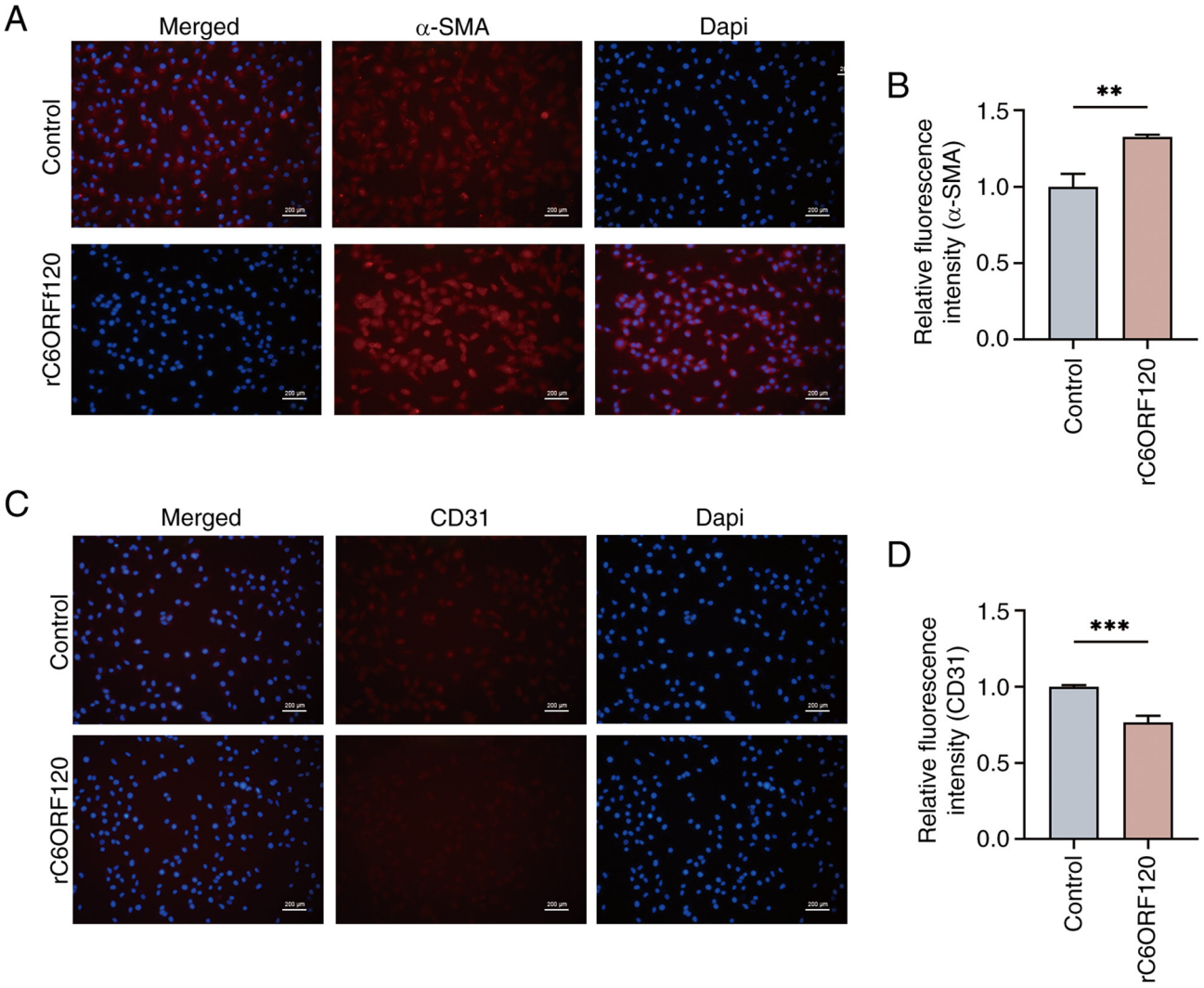

Figure 3. rC6ORF120 alters the fluorescence intensity of EndMT markers. Immunocytofluorescence staining of (A) α-SMA and (C) CD31 in HUVECs following rC6ORF120 treatment, with (B and D) corresponding statistical analysis. **P<0.01; ***P<0.001. Scale bar, 200 μm. rC6ORF120, recombinant C6ORF120 protein; EndMT, endothelial-mesenchymal transition; HUVECs, human umbilical vein endothelial cells.

migratory capacity relative to endothelial cells, a characteristic that is often accentuated during EndMT (1). Cell migration analysis via wound-healing assay demonstrated significantly increased wound closure in rC6ORF120-treated HUVECs compared with control (66.16±6.13 vs. 37.69±2.74%; t=7.35, P=0.002), indicating enhanced migratory capability (Fig. 4A and B).

Considering that angiogenesis is frequently associated with cell migration and EndMT, the present study further investigated the influence of rC6ORF120 on HUVEC tube-forming ability. Tube formation assays further revealed that rC6ORF120 significantly promoted angiogenesis, reflected by increased numbers of vascular junctions (990.00±29.87 vs. 766.00±76.37; t=4.73, P=0.009) and greater total vessel length (96203.00±3354.89 μm vs. 77199.67±4684.88 μm; t=5.71, P=0.005) compared with control (Fig. 4C-E).

rC6ORF120 activates the PI3K/Akt signaling pathway in HUVECs. Building on our previous research that suggested C6ORF120 could affect the PI3K/Akt signaling pathway (16). This led us to hypothesize that rC6ORF120 might similarly regulate this pathway in HUVECs. WB results indicated that rC6ORF120 significantly increased phosphorylation of PI3K (1.57±0.18 vs. control 1.00; t=5.64, P=0.005) and Akt (1.55±0.09 vs. control 1.00; t=11.12; P<0.001), confirming activation of the PI3K/Akt signaling pathway in HUVECs (Fig. 5).

rC6ORF120 promotes EndMT in HUVECs via the PI3K/Akt signaling pathway. To further explore the mechanism underlying rC6ORF120-induced EndMT, cells were pretreated with the PI3K inhibitor LY294002 prior to rC6ORF120 exposure. WB analysis demonstrated significant reversal of EndMT marker alterations: CD31 (1.18±0.12 vs. 0.65±0.06; t=7.00; P<0.001) and VE-cadherin (1.48±0.21 vs. 0.74±0.07; t=5.92, P=0.004) expression increased, while α-SMA (0.83±0.14 vs. 1.37±0.15; t=4.52, P=0.011) and Vimentin (0.82±0.17 vs. 1.35±0.12; t=4.50, P=0.013) expression decreased in LY294002 + rC6ORF120-treated cells compared with cells treated with rC6ORF120 alone (Fig. 6). These results were further confirmed by immunofluorescence staining (Fig. 7), indicating PI3K/Akt pathway as a critical mediator of rC6ORF120-induced EndMT.

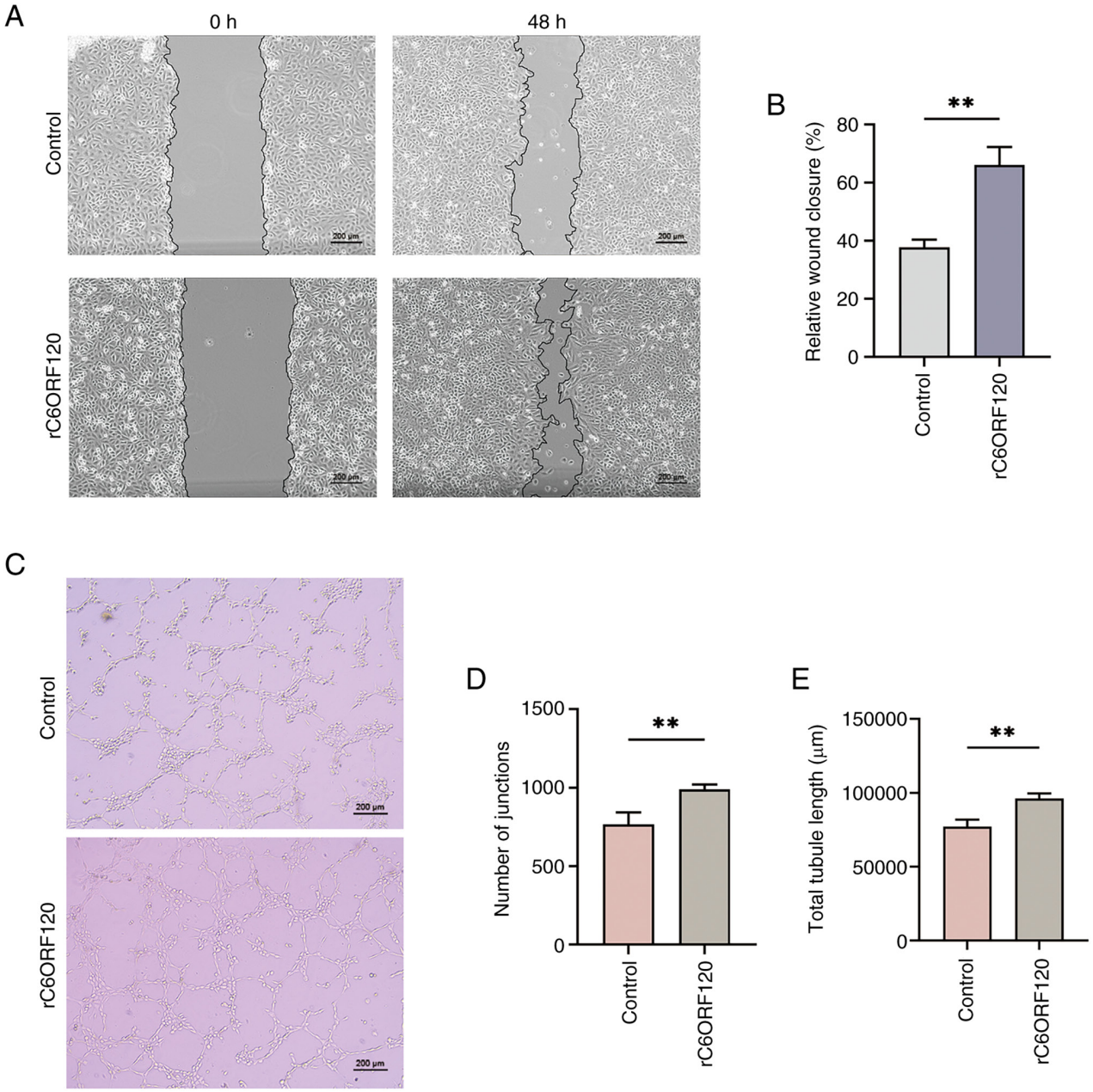

Figure 4. Effects of rC6ORF120 on the migration and angiogenic capacity of HUVECs. (A) Comparison of wound closure between the rC6ORF120-treated group and the control group after a 48-h incubation period. (B) Bar graph representing relative wound closure. (C) The impact of rC6ORF120 on the tube-forming capacity of HUVECs. Bar graph representing (D) number of junctions and (E) total tubule length. **P<0.01. Scale bar, 200 μm. rC6ORF120, recombinant C6ORF120 protein; HUVECs, human umbilical vein endothelial cells.

Differential role of PI3K/Akt pathway in rC6ORF120-mediated migration and angiogenesis. The present study further investigated the mechanisms underlying the influence of rC6ORF120 on HUVEC migration and angiogenesis. Notably, LY294002 did not significantly affect the migratory capacity (63.66±3.23% vs. 66.16±6.13%; t=0.63, P=0.565) (Fig. 8A and B) although LY294002 significantly reversed the angiogenic effects of rC6ORF120 (number of junctions: 709.67±55.64 vs. 990.00±29.87; t=7.69, P=0.002; total tubule length: 73579.33±4339.87 μm vs. 96203.00±3354.89 μm; t=4.76, P=0.009) (Fig. 8C-E). These findings indicated that while angiogenesis induced by rC6ORF120 depends heavily on PI3K/Akt signaling, migration enhancement probably involves alternative or redundant signaling pathways.

Discussion

Endothelial dysfunction plays a pivotal role in various pathological conditions and identifying key molecules and regulatory pathways involved in endothelial growth factor signaling may uncover novel therapeutic strategies for related diseases (32). Endothelial cells not only maintain vascular integrity as a mechanical barrier but also regulate critical physiological processes, including homeostasis, immune response and vascular tone (1). Endothelial dysfunction is increasingly recognized as a central pathological event underlying cardiovascular, metabolic and fibrotic disorders. The present study, for the first time to the best of the authors' knowledge, identified novel biological functions of the secreted protein C6ORF120 in endothelial cell

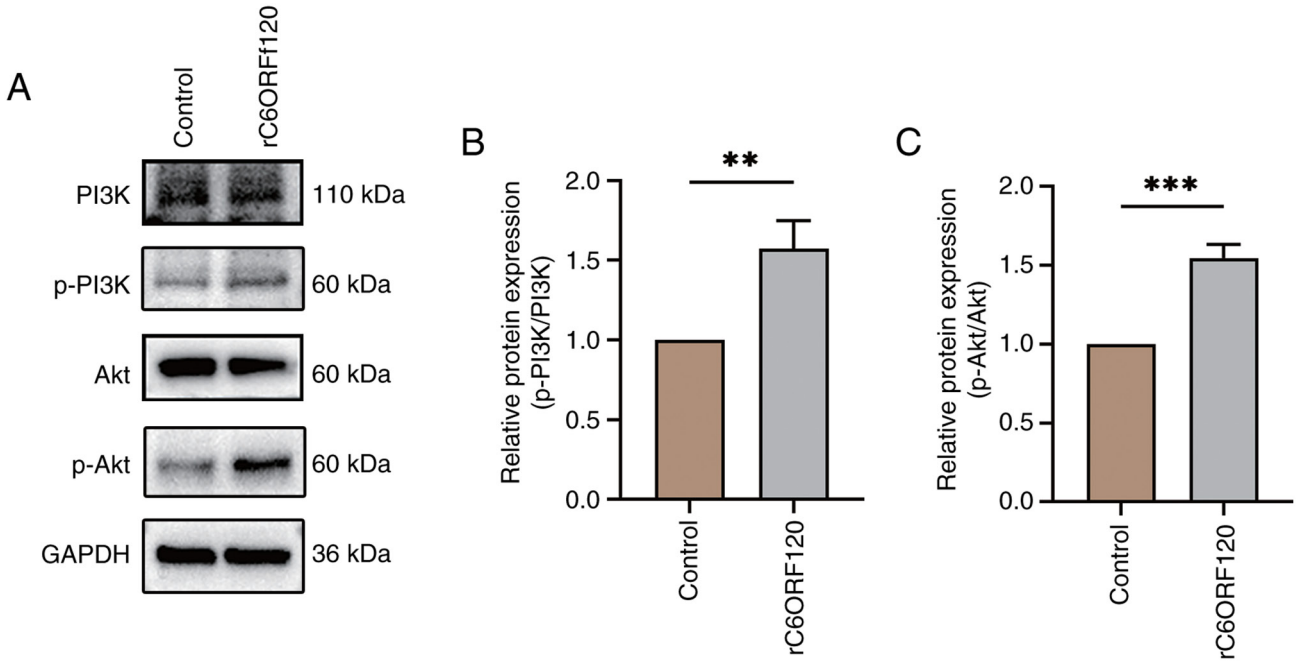

Figure 5. rC6ORF120 modulates the PI3K/Akt signaling pathway in HUVECs (A) Western blot analysis of PISK, p-PI3K, Akt and p-Akt in the HUVECs treated with rC6ORF120. Bar graph representing relative expression of (B) p-PI3K/PI3K and (C) p-Akt/Akt. **P<0.01; ***P<0.001. rC6ORF120, recombinant C6ORF120 protein; HUVECs, human umbilical vein endothelial cells; p-, phosphorylated.

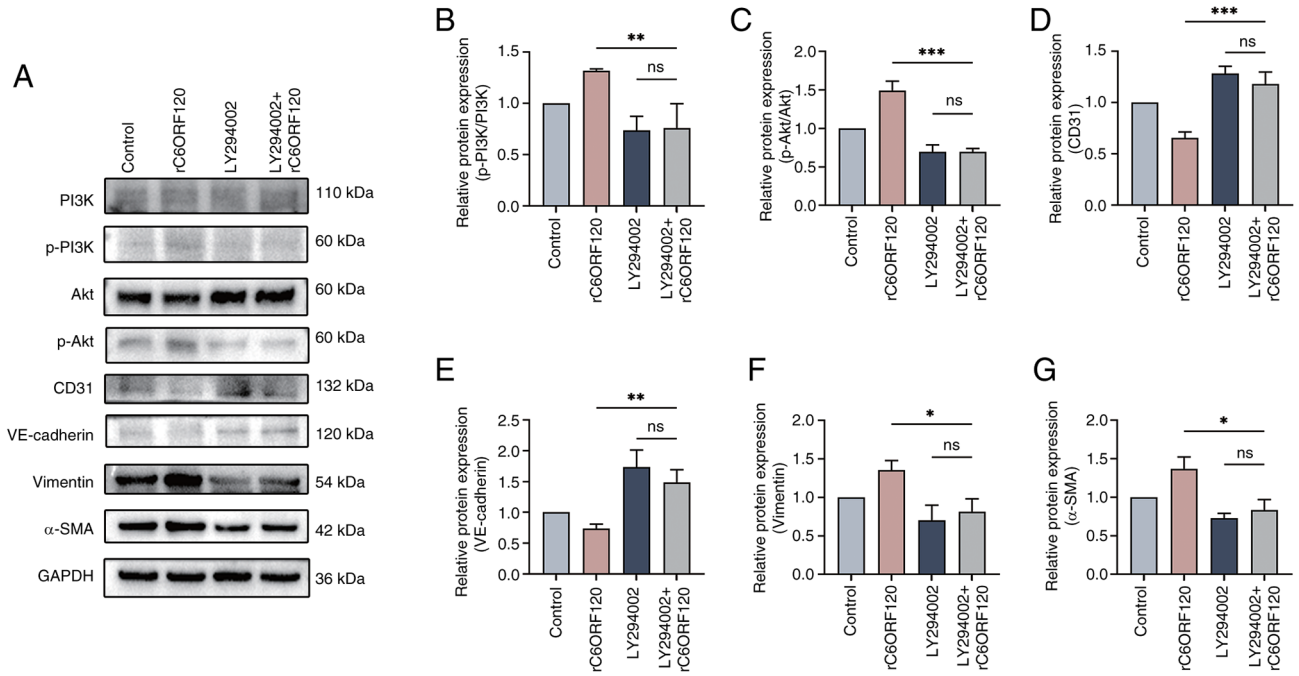

Figure 6. Involvement of the PI3K/Akt pathway in rC6ORF120-induced EndMT in HUVECs. Cells were treated with rC6ORF120, the PI3K inhibitor LY294002, or a combination of both. (A) Representative blots depicting the PI3K/Akt signaling pathway and proteins indicative of EndMT. (B-G) Quantitative assessment of protein expression levels. ns indicates no statistically significant difference; *P<0.05; **P<0.01; ***P<0.001. rC6ORF120, recombinant C6ORF120 protein; EndMT, endothelial-mesenchymal transition; HUVECs, human umbilical vein endothelial cells; p-, phosphorylated.

biology, demonstrating that rC6ORF120 promotes proliferation, migration, angiogenesis and EndMT in HUVECs, at least partly through activating the PI3K/Akt signaling pathway. This work is significant in directly establishing a link between C6ORF120 and endothelial dysfunction, thus broadening the functional repertoire of this protein.

The present study demonstrated that rC6ORF120 induced typical features of EndMT in HUVECs, including loss of endothelial markers (VE-cadherin and CD31), gain of mesenchymal markers (α-SMA and Vimentin), cytoskeletal remodeling and increased extracellular matrix production (33,34). EndMT is considered a form of cellular reprogramming, partial or

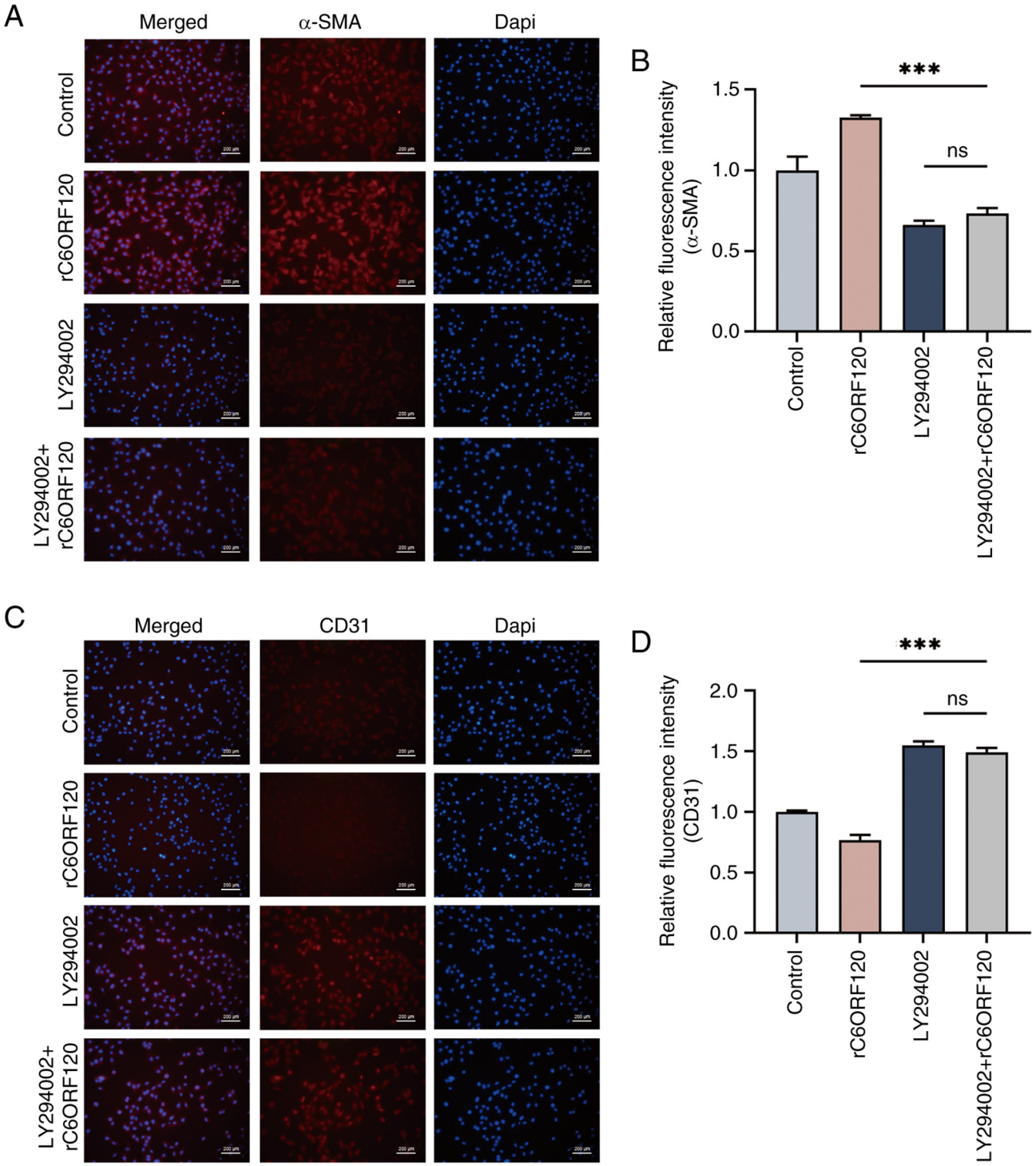

Figure 7. Inhibition of the PI3K/Akt pathway reverses the induction of EndMT by rC6ORF120. Immunofluorescence analysis of (A) α-SMA and (C) CD31. The bar graphs depict the relative fluorescence intensity of (B) α-SMA and (D) CD31. ns indicates no statistically significant difference; ***P<0.001. Scale bar, 200 μm. EndMT, endothelial-mesenchymal transition; rC6ORF120, recombinant C6ORF120 protein; α-SMA, α-smooth muscle actin.

complete, that enhances endothelial cell migratory and proliferative capacity (35,36). In line with this, it was also observed that rC6ORF120 significantly promoted migration and tube formation in HUVECs, highlighting its role in remodeling endothelial phenotype.

Mechanistically, our previous work suggested that C6ORF120 can modulate the PI3K/Akt pathway (16), which has also been implicated in regulating endothelial dysfunction in studies by Zhou *et al* (37) and He *et al* (38). The findings of the present study further identified rC6ORF120 as a novel upstream activator of PI3K/Akt signaling in HUVECs. Notably, pharmacological inhibition of this pathway with LY294002 effectively reversed rC6ORF120-induced EndMT and angiogenesis. However, the enhanced migratory capacity of HUVECs persisted, suggesting that rC6ORF120-mediated migration may be driven by PI3K/Akt-independent mechanisms.

Although endothelial migration and angiogenesis are functionally related, they are regulated by partially distinct

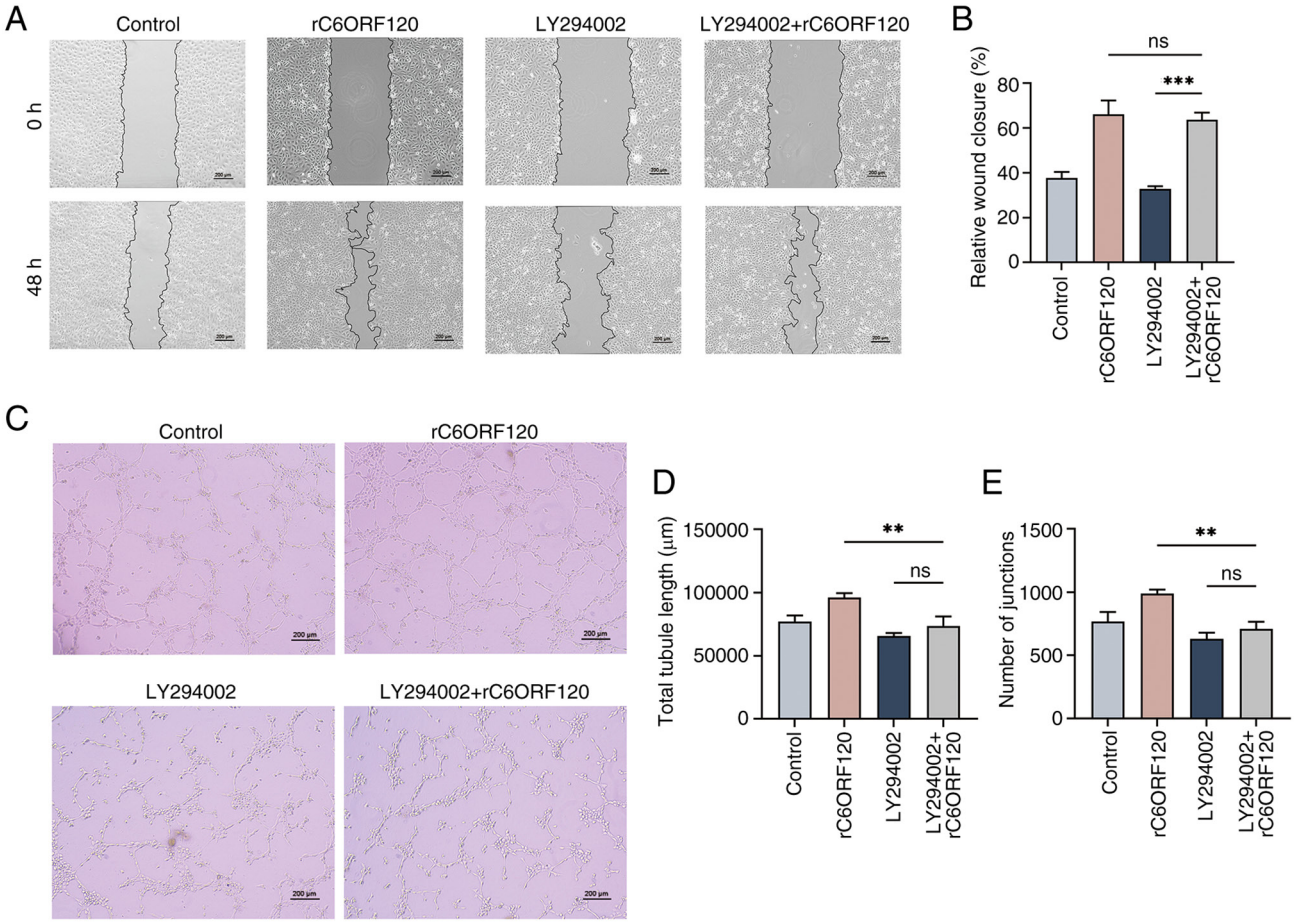

Figure 8. Effect of PI3K/Akt inhibition on rC6ORF120-mediated effects on HUVEC migration and angiogenesis. (A) Effect of rC6ORF120 on the migration of HUVECs after addition of LY294002. (B) Bar graph representing relative wound closure. (C) The reversing effect of LY294002 on rC6ORF120-induced angiogenic activity in HUVECs. Bar graph representing (D) number of junctions and (E) total tubule length. ns indicates no statistically significant difference; **P<0.01; ***P<0.001. Scale bar, 200 μm. rC6ORF120, recombinant C6ORF120 protein; HUVECs, human umbilical vein endothelial cells.

signaling programs. Angiogenesis is a complex multistep process involving matrix degradation, cell proliferation, migration, lumen formation and vessel maturation (39). Migration alone cannot complete this process; thus, distinct signaling controls each phase (40). The inability of LY294002 to inhibit rC6ORF120-induced migration suggests compensatory signaling involvement. Indeed, studies have shown that endothelial behavior is shaped by a network of partially redundant pathways, including MAPK, VEGF/VEGFR, Notch and Rho-GTPases (41-44). Moreover, in other cell types such as HepG2, the PI3K/Akt pathway modulates diverse processes (including autophagy, metabolism and oxidative stress) with function-specific variation in sensitivity (45,46). This could explain the functional divergence observed in our system.

As a secreted protein, rC6ORF120 may interact with multiple membrane receptors and initiate crosstalk across downstream signaling cascades, collectively influencing endothelial phenotype (47). For instance, class III semaphorins such as Sema3d modulate endothelial migration through distinct and parallel signaling routes involving PlxnD1 and Nrp1 (48). This supports the hypothesis that rC6ORF120 probably exerts its effects via multiple converging and compensatory signaling pathways. Future studies are needed to identify the key downstream effectors mediating rC6ORF120-induced migration, which may help clarify its broader regulatory network in endothelial dysfunction.

The present study offers two major innovations: Firstly, it provided the first evidence that rC6ORF120 regulates endothelial phenotype through both PI3K/Akt-dependent- and -independent mechanisms; and secondly it uncovered a previously unrecognized function of C6ORF120 in vascular biology beyond its known roles in immune modulation and fibrosis, suggesting its potential as a therapeutic target for vascular-related diseases. These findings contribute to a deeper understanding of the mechanisms driving endothelial dysfunction and may inform the development of new strategies for disease intervention.

Despite these strengths, several limitations should be noted. First, the present study is limited to *in vitro* experiments using HUVECs, which may not fully replicate the *in vivo* endothelial microenvironment. Second, while PI3K/Akt was identified as a major signaling mediator, other potential pathways such as MAPK, Rho/ROCK and Notch were not examined. Third, the specific receptors or upstream binding partners of rC6ORF120 remain unknown, which restricts comprehensive pathway mapping. Lastly, the long-term effects of rC6ORF120-induced EndMT and angiogenesis were not evaluated in animal disease models.

The present study provided novel insights into the functional role of C6ORF120 in endothelial biology and

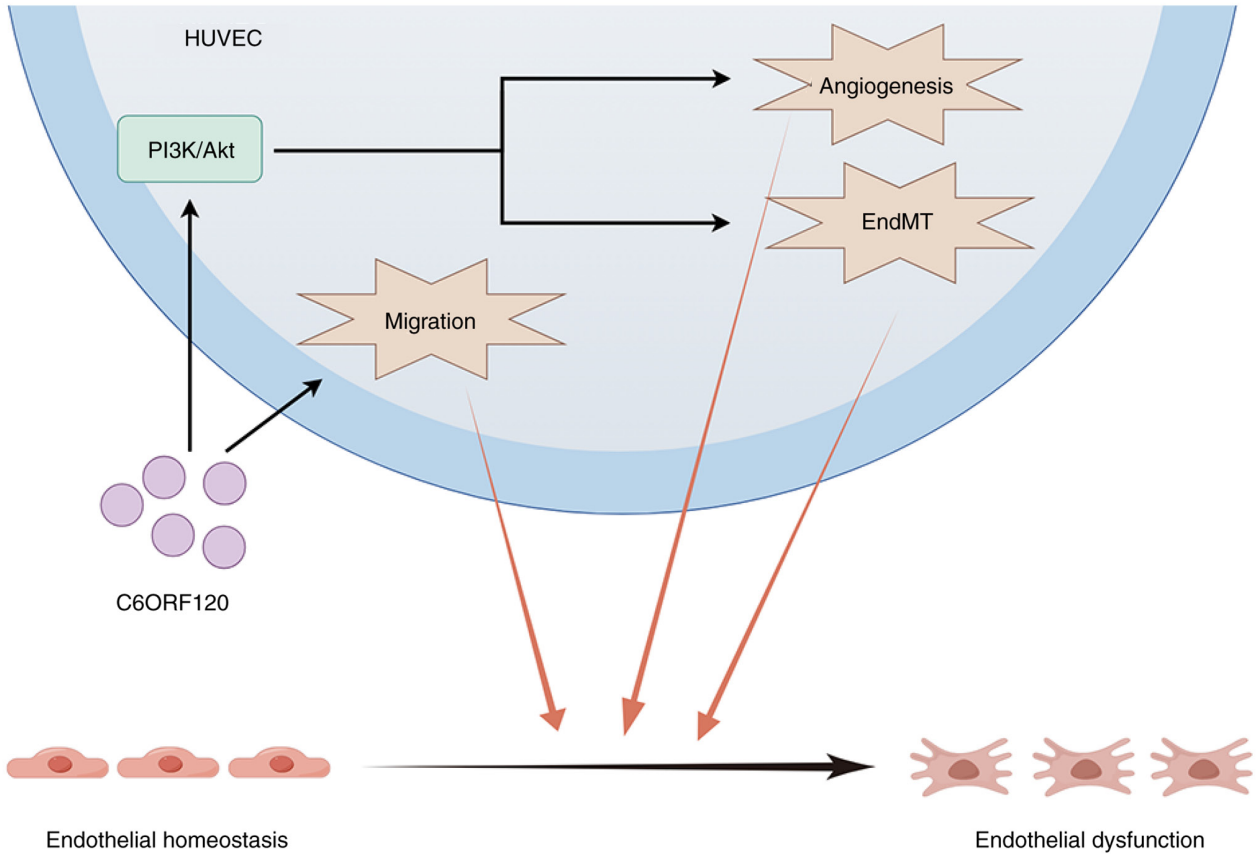

Figure 9. C6ORF120 enhances HUVEC migration, tube formation and angiogenesis, which are hallmark features of endothelial dysfunction. These processes are mediated, at least in part, by the PI3K/Akt signaling pathway. rC6ORF120, recombinant C6ORF120 protein; HUVECs, human umbilical vein endothelial cells; EndMT, endothelial-mesenchymal transition.

highlight its potential translational relevance. The ability of rC6ORF120 to induce EndMT and promote angiogenesis suggests its involvement in pathological vascular remodeling, which underlies a broad spectrum of diseases, including atherosclerosis, hypertension, fibrosis and tumor progression. Given that C6ORF120 is a secreted protein, it holds promise not only as a biomarker for endothelial plasticity but also as a therapeutic target for modulating vascular dysfunction. In particular, targeting C6ORF120 or its downstream PI3K/Akt signaling axis may offer novel strategies for preventing or reversing endothelial maladaptation in cardiovascular and fibrotic disorders. Future studies evaluating circulating C6ORF120 levels and functional inhibition in *in vivo* disease models are warranted to further define its clinical utility.

In conclusion, the present study identified rC6ORF120 as a novel regulator of endothelial dysfunction, capable of promoting EndMT, angiogenesis and migration through both PI3K/Akt-dependent and independent pathways. These findings expanded the functional spectrum of C6ORF120 and provided new insights into endothelial regulation with potential therapeutic implications, thereby opening new avenues for research in vascular biology and translational medicine.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YiL, XW and XL conceived the study, designed the methodology and performed the formal analysis. YaL, XC and NZ conducted the investigation. YiL and XW prepared the original draft, while YaL, XC, NZ and XL reviewed and edited the manuscript. XL managed the project. XL secured funding. YiL, XW and XL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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