

Received: 2019.10.21

Accepted: 2019.12.13

Available online: 2020.01.21

Published: 2020.03.02

Spalt-Like Protein 4 (SALL4) Promotes Angiogenesis by Activating Vascular Endothelial Growth Factor A (VEGFA) Signaling

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Source of support: This work was supported by the National Natural Science Foundation of China (81772745) and the Science and Technology Innovation Development Foundation of Fourth Military Medical University (2016XB078)

Background: Spalt-like protein 4 (SALL4) is a nuclear transcription factor central to early embryonic development, especially for regulating pluripotency of embryonic stem cells (ESCs) and sustaining ESCs self-renewal. Aberrant re-expression of SALL4 in adult tissues is involved in tumorigenesis and cancer progression. However, the role of SALL4 in angiogenesis remains elusive. Here, we determined the potential action of SALL4 on proliferation, migration, and tube formation of endothelial cells.





Material/Methods: HUVECs were infected with lentiviral particles expressing shRNA against SALL4. QRT-PCR and immunoblotting analysis were carried out to evaluate knockdown efficiency at mRNA and protein levels. Cell proliferation was measured by CCK-8 assay and flow cytometry was conducted to analyze cell cycle distribution. Wound-healing and Transwell migration assays were performed to evaluate cell motility. In addition, we determined the role of SALL4 on angiogenesis by tube formation assay, and Western blot analysis was used to assess the effect of SALL4 downregulation on VEGFA expression.

Results: We found that SALL4 downregulation resulted in decreased proliferation. Cell cycle analysis revealed that SALL4 knockdown impeded cell cycle progression and induced cell cycle arrest at G1 phase. We also found that silencing of SALL4 decreased the capacity of wound healing and cell migration in HUVECs. Furthermore, tube formation assay showed that loss of SALL4 inhibited HUVECs angiogenesis. We also observed that SALL4 knockdown reduced the level of VEGFA in HUVECs.

Conclusions: In conclusion, these results support that by promoting proliferation, cell cycle progression, migration, and tube formation, SALL4 is involved in the process of angiogenesis through modulating VEGFA expression.

MeSH Keywords: **Angiogenesis Inducing Agents • Cell Migration Assays • Cell Proliferation • Vascular Endothelial Growth Factor A**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/920851>

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Background

The vascular network conducts blood flow to supply nutrients, oxygen, and growth factors, and removes metabolic waste. Sprouting angiogenesis is a process that new blood vessels develop from pre-existing vasculature [1,2]. Neovascularization during embryonic development is a combined process of angiogenesis and vasculogenesis, a process of vessels formation from angioblast precursor [3,4]. Angiogenesis can be triggered by the events of embryogenesis and tissue repair [2]. Abnormal angiogenesis has been reported to be involved in the pathogenesis of various diseases, such as cancer. Over the past decades, tremendous scientific breakthroughs have been made in illuminating the signaling pathways that play critical roles in angiogenesis. Accumulating evidence has implicated vascular endothelial growth factor (VEGF) signaling in the regulation of vasculature growth and morphogenesis during physiological and pathological neovascularization. Extensive research into the mechanisms of angiogenesis has led to multifaceted therapeutic approaches targeting certain phases of the process, especially VEGF signaling, either to improve vascularization and tissue perfusion for ischemic diseases [5,6] or to block blood flow supply to curb growth and normalize the impaired vasculature for facilitating drug delivery in treatment of cancers [7,8].

SALL4 (spalt-like protein 4), a transcription factor containing zinc-finger domains, plays a critical role in regulating pluripotency of embryonic stem cells (ESCs) and sustaining ESCs self-renewal [9]. Three SALL4 isoforms, termed A, B, and C, have been reported to exist in both humans and mice [10,11]. SALL4B and C isoforms are 2 products from alternative splicing of SALL4A. SALL4A and SALL4B can bind to distinct DNA binding sites as homodimers or heterodimers and collaborate in maintaining ESCs pluripotency [10]. Human mutation of SALL4 can result in a genetic disorder characterized by defects in limbs and heart [12]. SALL4 is indispensable for embryogenesis, but is rarely expressed in most adult tissues. However, aberrant expression of SALL4 is observed in numerous malignancies, such as leukemia, liver cancer, gastric cancer, and cervical cancer [11–16]. The re-expressed SALL4 is shown to be responsible for tumor growth, metastasis, and treatment resistance through activating multiple signaling pathways, such as PI3K/Akt and Wnt/ β -catenin pathways [13,16]. It is well established that vasculogenesis and angiogenesis are required for embryogenesis and critically regulate multiple physiological processes during embryonic development. In addition, enhanced vascularization in cancers has been reported to be associated with poor patient prognosis, and well-vascularized tumors have enormous proliferative capacity and metastatic potential [17]. Furthermore, recent studies have demonstrated that PI3K/Akt and Wnt/ β -catenin pathways are involved in the modulation of angiogenic processes via crosstalk with HIF-1 α /VEGF

signaling [18,19], suggesting that divergent intracellular signaling can converge, in part to regulate a common biological process. All the evidence mentioned above has led us to investigate the relationship between SALL4 and angiogenesis.

In the present study, we explored the potential role of SALL4 in regulation of angiogenic processes. We found that SALL4 was involved in endothelial cell proliferation, migration, and tube formation. We also found that SALL4 exerts its effects on angiogenesis via VEGF signaling. Delineation of the effects of SALL4 on molecular pathways involved in angiogenesis could be conducive to the translation of novel therapeutic approaches.

Material and Methods

Cell culture

The human umbilical vein endothelial cell line HUVEC was purchased from ATCC and maintained in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12, Gibco) with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL) (Gibco). HUVECs were cultured in a chamber (37°C) with 5% CO₂. Trypan blue exclusion test for cell viability was performed prior to experiments.

Lentiviral infection

SALL4 short hairpin RNA (shRNA) was used for stable SALL4 downregulation in HUVECs. The packaging of short hairpin RNA (shRNA) lentiviruses was performed by Obio Technology (Shanghai, China). The target sequences of shRNAs (SALL4-targeted shRNA, shSALL4; negative control, shNC) were as follows:

shSALL4, 5'-GCCTTGAACAAGCCAAGCTA-3'; and
shNC, 5'-TTCTCCGAACGTGTCACGT-3'.

HUVECs were infected with viral supernatant and then cultured in medium supplemented with puromycin (2.5 μ g/mL) for selection of stable transfections according to the instructions of the manufacturer.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and purified from HUVECs using TRIzol reagent (Invitrogen) according to protocols of the manufacturer. PrimeScript™ RT Master Mix (TaKaRa) was used to reverse-transcribe the extracted RNA for cDNA synthesis. The cDNA was then subjected to qRT-PCR analyses using SYBR® Premix ExTaq™ II (TaKaRa) on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA). The fold changes of mRNA were determined using 2^{- $\Delta\Delta$ CT} method. Beta-actin was set as a normalization control. The qRT-PCR primers were as follows:
 β -actin forward, 5'-TGGCATCCACGAACTACC-3';

reverse, 5'-GTGTTGGCGTACAGGTCTT-3';
SALL4 forward, 5'-ATCCGCATCCAGGTGAACAT-3';
reverse, 5'-TCAAGGCATCCAGAGACAGAC-3'.

Western blotting

RIPA buffer supplemented with a cocktail of protease inhibitor was used to extract proteins for immunoblotting. After quantifying the concentrations via BCA method (Thermo), the lysates were subjected to SDS-PAGE for separation. After proteins were transferred onto a polyvinylidene fluoride membrane (Millipore), the membrane was blocked in 5% nonfat milk at room temperature for 1 h. Next, the membrane was probed using primary antibodies (anti-SALL4, 1: 1000 dilution, Cell Signaling Technology #5850; anti-VEGFA, 1: 500 dilution, Sangon Biotech #D160788; anti-GAPDH, 1: 2000 dilution, Sangon Biotech #D110016) overnight at 4°C. After incubation with HRP-linked anti-rabbit secondary antibody (1: 5000 dilution, Jackson ImmunoResearch #124791), immunoreactive bands were detected using enhanced chemiluminescence substrate (Millipore) following protocols of the manufacturer.

Cell proliferation assay

Cell Counting kit-8 (CCK-8) (Sigma-Aldrich) assay was conducted to measure proliferation ability following the protocols of the manufacturer. Briefly, HUVECs were plated into 96-well plates (2000 cells per well) in triplicate. CCK-8 solution (20 µl) was added to the medium for indicated times. After incubation for 1 h at 37°C, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad). Three independent experiments were carried out.

Cell cycle analysis

Flow cytometry analysis was performed to determine the effect of SALL4 on cell cycle distribution of HUVECs. HUVECs were collected and washed 3 times with PBS. After fixation in 70% ethanol overnight at 4°C, 1×10^6 cells were incubated with RNase A (100 µg/mL) for 1 h at 37°C, followed by incubation with staining solution containing propidium iodide (50 µg/mL) for 15 min at room temperature. The HUVEC group with serum-free medium (SFM) treatment was used as a positive control. The DNA contents in cells with indicated treatments were analyzed by flow cytometry (BD Biosciences).

Wound-healing assay

We plated 5×10^5 HUVECs in 6-well plates and grew them to almost 100% confluence. A scratch was made using a sterile 200-µl pipette tip. The cells were washed and incubated with serum-free medium. The HUVEC group without shRNA treatment was used as a blank control. Wound closure was

observed by evaluating the distance of cell migration and imaged under a microscope (100x magnification) at 0, 24, and 48 h after scratching.

Transwell migration assay

To assess cell migration ability by Transwell assay, 1×10^5 HUVECs suspended in 200 µl of DMEM/F-12 without FBS were seeded onto the upper chamber (8-µm pore size, 24-well insert, Millipore) without Matrigel coating, and the lower chamber received 500 µl of DMEM/F-12 containing 10% FBS. After incubation for 24 h, the cells in the upper chamber were gently removed using a cotton swab. The migrated cells on the lower chamber membrane surface were fixed with 4% paraformaldehyde and stained with crystal violet. Then, the cells were photographed and counted with a manual cell counter under microscopy at 200x magnification.

Tube formation assay

HUVECs (5×10^4) were plated into a 96-well plate coated with Matrigel (BD Biosciences) and incubated in normal growth medium for 6 h at 37°C. The tube-like structures were observed and photographed under a light microscope at 200x magnification. Analysis of tube formation was carried out using Angiogenesis Analyzer plugin in ImageJ software (version 1.48, National Institutes of Health).

Statistical analysis

SPSS 18.0 software (SPSS, Inc.) was used for statistical analyses. Data collected from 3 independent repetitions are presented as mean±SD or mean±SEM. The *t* test was used to evaluate differences between 2 groups. One-way ANOVA was used to analyze differences among more than 2 groups. Statistical significance (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) was indicated.

Results

Downregulation of SALL4 suppresses HUVEC proliferation and cell cycle progression

To investigate the potential role of SALL4 in endothelial growth *in vitro*, we established HUVECs with stable downregulation of SALL4 (shSALL4) or negative control (shNC) via lentiviral transduction. Fluorescence microscopy analyses showed that the percentage of EGFP-positive cells was more than 90% (Figure 1A). Additionally, qRT-PCR analysis indicated that the mRNA level of SALL4 was significantly downregulated in shSALL4-treated HUVECs compared with that of shNC-treated cells (Figure 1B). Further analysis by Western blot revealed that, in comparison with the HUVEC-shNC group, the protein levels of both SALL4A

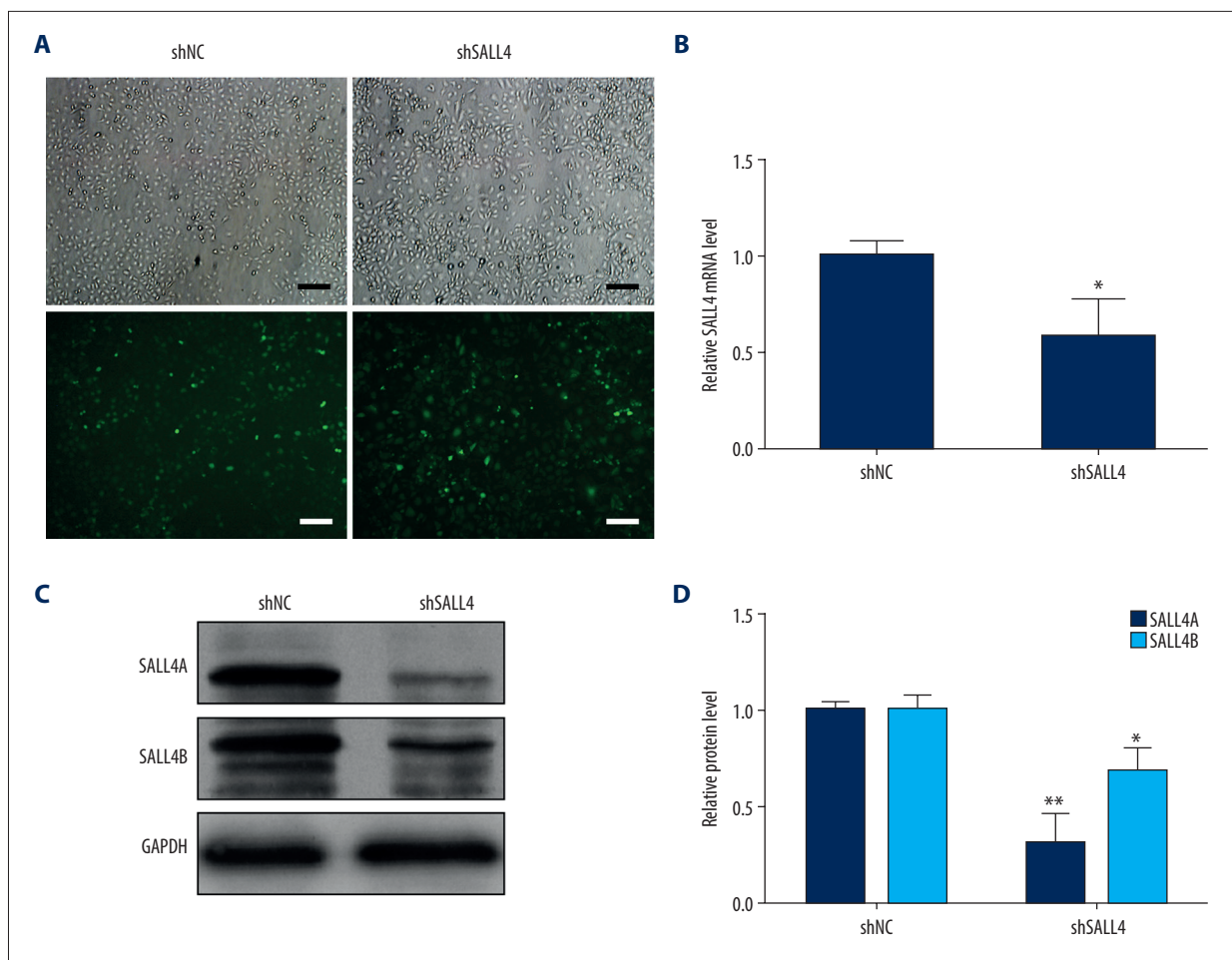


Figure 1. Efficiency of lentivirus-mediated SALL4 knockdown in HUVECs. **(A)** HUVECs were treated with virus supernatant for 72 h and pictures (100×) were taken to evaluate the infection efficiency. Scale bar=200 μm. **(B)** qRT-PCR analysis of SALL4 mRNA level in stably infected cells. **(C)** Western blot analysis of SALL4 protein level in stably infected cells. **(D)** Quantification of SALL4 protein level in western blot analyses. * $P < 0.05$ and ** $P < 0.01$ vs. shNC (Student's *t*-test).

and SALL4B were remarkably decreased in the HUVEC-shSALL4 group (Figure 1C, 1D). To determine whether SALL4 is critically involved in endothelial proliferation, stable transfections of HUVEC was subjected to CCK-8 assay. We found that knockdown of SALL4 resulted in significant decrease in proliferation of HUVECs compared with the control group (Figure 2A). To further verify the growth-promoting action of SALL4 in endothelial cells, flow cytometry analysis was performed to evaluate the influence of SALL4 downregulation on cell cycle progression. We observed a notable alteration in cell cycle distribution. The data demonstrated that SALL4 knockdown led to a significant increase in the proportion of G1-phase cells and a remarkable decrease of cells in G2 phase, when compared with the shNC group (Figure 2B, 2C), indicating an arrested cell cycle at G1 phase. Taken together, these findings suggest that SALL4 plays a pivotal role in endothelial growth.

Downregulated SALL4 expression represses cell migration of HUVECs

The migratory ability of endothelial cells is a critical characteristic of angiogenesis. To explore whether SALL4 plays a significant role in endothelial cell migration, we first performed the scratch wound-healing assay to determine the effect of SALL4 inhibition on HUVECs. Our results showed that SALL4 knockdown significantly prevented HUVECs from forming a monolayer of cells to heal the wound area at 24 h and 48 h when compared to the control cells (Figure 3A–3C), indicating impaired migratory ability by loss of SALL4. To further validate the above results, we performed additional Transwell migration assays to measure the effect of decreased SALL4 level on HUVECs migration. We found that downregulating SALL4 expression markedly retarded HUVECs migration from the upper chamber to the lower surfaces of the chamber membrane compared with the control cells (Figure 3D, 3E). Collectively,

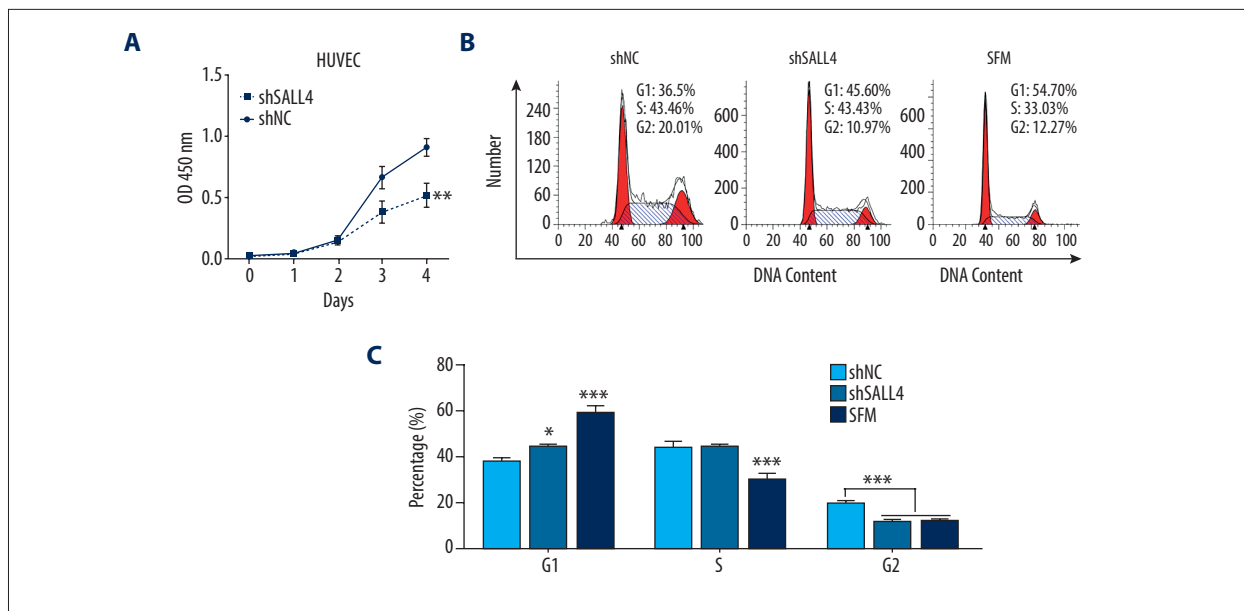


Figure 2. SALL4 downregulation inhibits endothelial cell growth. (A) Effect of SALL4 knockdown on cell proliferation was determined by CCK-8 assay in HUVECs. *** $P < 0.001$ vs. shNC (t test). (B) Cell cycle distribution was detected by flow cytometry in HUVECs treated with shRNAs and serum-free medium (SFM). (C) Statistical analysis of cells in different phases. * $P < 0.05$ and *** $P < 0.001$ vs. shNC (one-way ANOVA).

these results demonstrate that SALL4 is involved in the regulation of endothelial cell migration.

Knockdown of SALL4 results in reduced tube formation of HUVECs

Given that downregulation of SALL4 significantly suppressed endothelial cell proliferation and migration, we then attempted to determine whether SALL4 affects tubular structure formation. To further verify the significant function of SALL4 in angiogenesis, we performed capillary-like tube formation assays to characterize the effect of SALL4 inhibition. We found that fewer tubular structures were formed when SALL4 was downregulated in HUVECs (Figure 4A, 4B). Additionally, our data revealed that SALL4 knockdown led to reduced ability of tube formation in terms of decreased branch points and tube length (Figure 4C, 4D). Considering the prominent role of HIF-1 α /VEGF signaling in angiogenesis, we thus wanted to investigate whether SALL4 affected this signaling in HUVECs. Western blot analysis demonstrated that downregulation of SALL4 expression resulted in significant decrease of VEGFA protein level (Figure 4E, 4F), whereas no remarkable alteration in HIF-1 α expression was observed (data were not shown). Together, these results suggest that SALL4 promotes angiogenesis by manipulating VEGFA signaling.

Discussion

Here, we investigated the potential role of SALL4 in angiogenesis. We found that SALL4 downregulation was associated with suppressed cell proliferation, cell cycle progression, migration, and capillary-like tube formation in HUVECs. Further, SALL4 level was positively correlated with the expression of VEGFA, which may be critical for the role of SALL4 in HUVECs.

Angiogenesis drives new blood vessels formation from existing ones through sprouting. It is an important process that is not only involved in physiological conditions such as development and tissue homeostasis, but is also associated with numerous pathological conditions, including inflammation and tumorigenesis. This process consists of several steps featuring different endothelial cell functions, including cell differentiation, migration, proliferation, lumen formation, and maturation [20]. Previous studies have shown that numerous angiogenic factors, receptors, and signaling pathways participate in modulating new blood vessel formation and morphogenesis [5].

SALL4, a zinc-finger transcriptional factor, plays multiple roles in embryonic development and cancer progression [9,13,21], in which angiogenesis is also an indispensable player. SALL4 has been demonstrated to participate in the development of the inner cell mass, and SALL4-deficient mice rarely survive beyond embryonic day 6.5 [22]. Similarly, defective proliferation was observed in inner cell mass cells *in vitro* [23]. One study showed that SALL4 was necessary for efficient proliferation

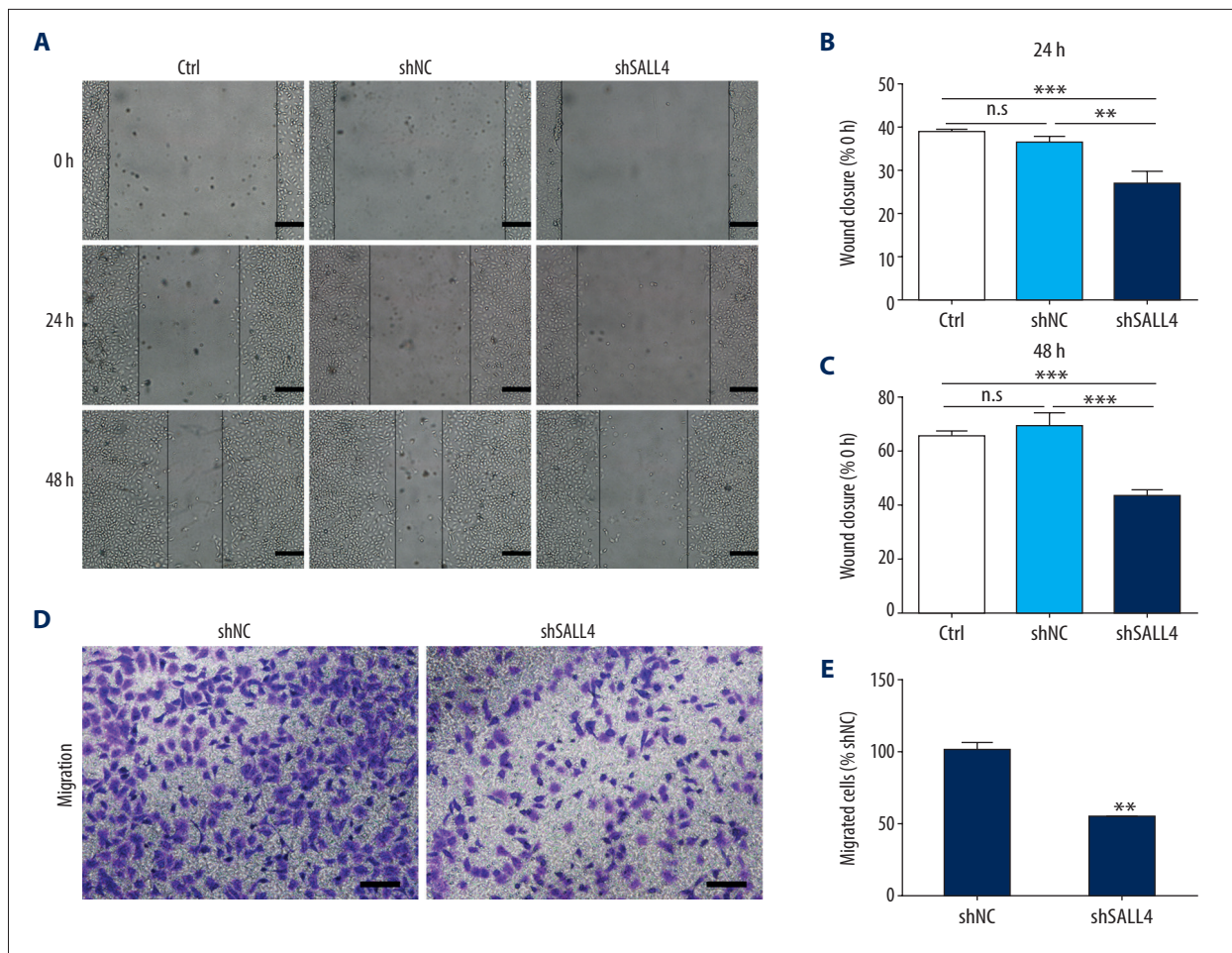


Figure 3. SALL4 knockdown suppresses endothelial cell motility *in vitro*. (A) HUVECs treated with or without shNC/shSALL4 were subjected to wound-healing assays. Scale bar=200 μ m. Statistical analysis of wound closure at 24 h (B) or 48 h (C). n.s. – no significance, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA). (D) HUVECs treated with shNC/shSALL4 were subjected to Transwell migration assays. Scale bar=100 μ m. (E) Quantification of cells on the lower chamber membrane surface. ** $P < 0.01$ vs. shNC (*t* test).

of embryonic stem cells co-cultured with mouse embryonic fibroblast feeder cells by suppressing trophoblast gene expression [24]. Furthermore, previous evidence indicates that SALL4 is critical for cell proliferation and apoptosis in cancers. SALL4 downregulation results in significant cell cycle arrest and growth inhibition in human leukemic cells [15]. In addition, loss of SALL4 markedly decreases human leukemia cells xenograft growth in immunodeficient mice. In agreement with the above findings, overexpression of SALL4 promotes cell proliferation and cell cycle transition from G1 to S phase via upregulating the levels of cyclin D1 and D2 in nasopharyngeal carcinoma and cervical cancer, while downregulated SALL4 leads to suppression of tumor growth *in vitro* and *in vivo* [16,25]. To support the highly proliferative cancer cells, tumors need to continuously develop new blood vessel networks to have access to oxygen and nutrients. Considering the effects of angiogenesis in embryonic development and cancer progression, SALL4

may play a role in vascularization. In support of the role for SALL4 driving endothelial cell growth, we observed that a low SALL4 level is predictive of decreased cell proliferation and delayed cell cycle progression in HUVECs.

Prior evidence indicates that SALL4 levels are positively correlated with lymph node metastasis in human gastric cancer. Forced expression of SALL4 in gastric cancer cells improves the cell migration ability, whereas SALL4 knockdown results in the opposite effect [14]. In endometrial cancer and esophageal squamous cell carcinoma, similar effects have been observed both *in vitro* and *in vivo* [26,27]. Further studies have implicated SALL4 in modulating the expression of E-cadherin, Twist1, and ZEB1, all of which are markers of epithelial-mesenchymal transition (EMT). Therefore, SALL4 may be involved in tumor metastasis via induction of EMT. Metastasis, a major form of cancer progression, is a highly complex process.

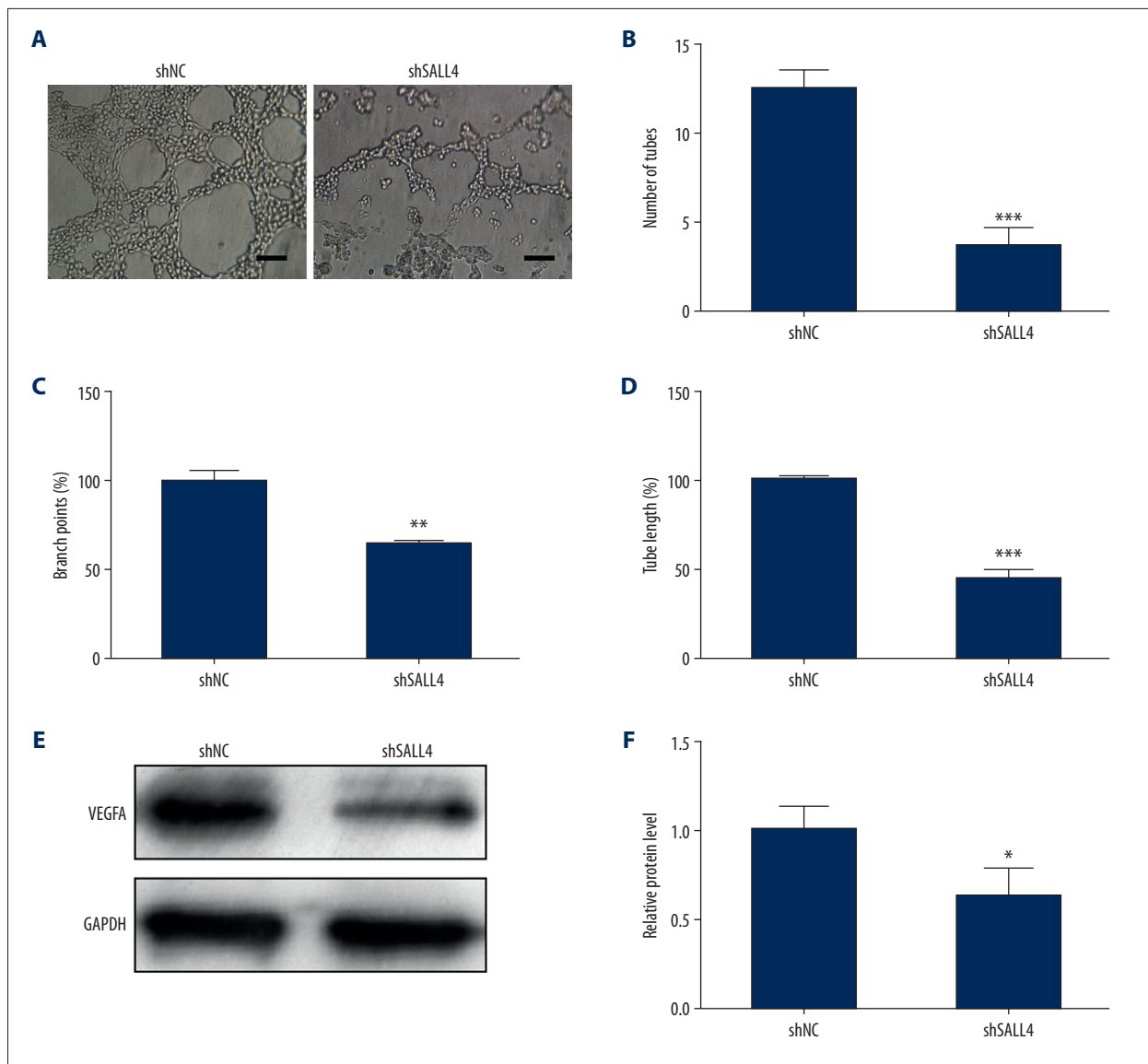


Figure 4. Silencing of SALL4 impedes endothelial cell tube formation via targeting VEGFA. (A) Angiogenesis ability was measured by performing tube formation assays in shNC/shSALL4-treated HUVECs. Scale bar=100 μ m. Statistical analyses of tube numbers (B), branch points (C), and tube length (D) per microscopic field were performed. (E) Western blot analysis of VEGFA expression in shNC/shSALL4-treated HUVECs. (F) Statistical analysis of VEGFA protein level in Western blot analyses. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. shNC (*t* test).

Establishment of angiogenic blood vessels is required to support tumor metastasis. The metastasis-supporting neovascu- lature can serve as an intermediary to provide both the nutri- ents for invasive tumor growth and the conduits for escaping tumor cells dissemination to secondary sites [17]. Considering the functional roles of SALL4 and neovascularization in tumor metastasis, it is conceivable that SALL4 may be linked to angiogenesis. In support of the role for SALL4 in angiogenesis, we observed that SALL4 knockdown decreases the capacity of cell migration in HUVECs.

Particular stimuli, such as hypoxia, trigger the angiogenesis switch. A local imbalance between angiogenic and angiostatic factors are created, which results in establishment of a vas- cular supply. Vascular growth is modulated by multiple angio- genesis-associated signals, among which, the HIF-1 α /VEGFA pathway plays critical roles in initiation and promotion of angio- genesis. Under hypoxic conditions, HIF-1 α escapes from VHL-mediated degradation and accumulated HIF-1 α proteins bind to hypoxia response elements, thus activating the tran- scription of downstream target genes that are critical for cel- lular adaptation to a limited concentration of oxygen [28].

These genes, such as VEGF, TGF- β , and PDGF-B, are involved in endothelial cell proliferation, migration, metabolism, and angiogenesis [29].

Crosstalk between HIF-1 α /VEGF and other molecular pathways has been validated to exist at multiple levels. It was reported that activation of MEK/ERK signaling promotes the translation of HIF-1 α mRNA [30,31]. Additionally, EGFR-mediated activation of the PI3K/Akt pathway has been implicated in angiogenesis via modulating VEGF expression, and PI3K inhibition decreases the levels of VEGF [32]. Furthermore, the anomalous activation of Wnt/ β -catenin induces metabolic remodeling through Wnt target genes or HIF-1 α overexpression, and are correlated with enhanced vasculogenesis and angiogenesis, even under normoxic conditions [33]. Previous studies demonstrated that, transcriptionally activated by canonical Wnt signaling, SALL4 in turn can be complexed with β -catenin to collaboratively activate the transcription of Wnt target genes [16,19], thus establishing a positive feedback loop. A mounting body of evidence indicates that homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K/Akt signaling, is a downstream target of SALL4 [13]. SALL4 can recruit nucleosome remodeling deacetylase (NuRD) to the promoter region to repress PTEN transcription [34]. A therapeutic peptide targeting the SALL4-NuRD interaction abolishes the transcription-repressor action of SALL4 [13,35], which results in restoration of PTEN expression and decreased PI3K/Akt signaling. We thus infer that SALL4 may play an important role in angiogenic process through modulation of HIF-1 α /VEGF signaling. Indeed, we observed that HUVECs expressing low levels of SALL4 exhibit impaired tube formation ability, and deletion of SALL4 in HUVECs results in reduced protein level of VEGFA with no significant change in HIF-1 α expression, indicating that SALL4 may regulate VEGFA expression through activation of PI3K/Akt or Wnt/ β -catenin pathways.

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In summary, our results demonstrate that SALL4 promotes endothelial cell growth, migration, and capillary-like tube formation via targeting VEGFA. The specific role for SALL4 in the regulation of VEGF activity requires further investigation. Whether SALL4 is involved in other molecular pathways relevant to angiogenesis remains to be determined in future studies. In-depth understanding of SALL4 molecular mechanisms promoting angiogenesis will lead to new therapeutic strategies for some diseases that are induced by aberrant SALL4 expression.

Conclusions

Taken together, the results in this study indicate that SALL4 plays a critical role in angiogenesis. Downregulation of SALL4 suppresses endothelial cell proliferation, cell cycle progression, migration, and tube formation through targeting VEGFA. Therefore, modulating SALL4 may be an effective strategy for prevention and treatment of certain diseases that are closely involved with angiogenesis. However, further exploration is needed to elucidate whether SALL4 directly targets VEGFA or other angiogenic signaling molecules, and the detailed mechanisms involved in SALL4-mediated angiogenesis require confirmation by additional *in vitro* and *in vivo* experiments.

Acknowledgements

We would like to thank the technical support we received from the Medical Laboratory and Research Center, Tangdu Hospital, Fourth Military Medical University.

Conflict of interest

None.

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