

## Original Article

# Effects of survivin on angiogenesis *in vivo* and *in vitro*

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**Abstract:** Objective: In this study, the effects of survivin (SVV) on angiogenesis were evaluated *in vitro* and *in vivo*. Methods: The adenovirus (Ad)-mediated murine SVV gene was transfected into rat aortic endothelial cells (RAECs). RAECs expressing green fluorescent protein after transfection with Ad served as a negative control and those without transfection as a blank control. Then, the SVV mRNA was detected by quantitative real time RT-PCR. The SVV protein, cell cycle and apoptosis related proteins, and matrix metalloproteinase (MMPs) were detected by western blot assay. Immunofluorescence staining was conducted for proliferating cell nuclear antigen and MTT assay for cell viability. Transwell and matrigel chamber assay were employed to assess the migration and invasion of cells after transfection. TUNEL staining and flow cytometry were performed to detect the apoptotic REACs after treatment with anti-Fas antibody. Tube formation in matrigel membranes and matrigel plugs assay in nude mice were employed to confirm the angiogenic capacity *in vitro* and *in vivo*, respectively. Results: The mRNA and protein expressions of SVV increased significantly in SVV transfected cells. The SVV transfected cells showed increased cell proliferation, up-regulated expressions of cell cycle proteins, enhanced invasiveness and migration activities and increased expressions of MMP-2, 7 and 9. In addition, SVV protected against apoptosis of RAECs by inactivating caspase-3, 8 and 9. The tube formation and matrigel plugs assays showed SVV significantly increased blood vessels *in vitro* and *in vivo*. Conclusion: SVV may act as an angiogenic factor and used for therapeutic angiogenesis in peripheral arterial diseases.

**Keywords:** Survivin, therapeutic angiogenesis, peripheral arterial disease, rat aortic endothelial cell

## Introduction

Peripheral arterial diseases (PAD) are often caused by atherosclerosis, which results in the progressive narrowing and occlusion of the peripheral arteries and inhibits the blood flow to the lower extremities. Critical limb ischemia (CLI) represents a complication of PAD and is characterized by rest pain, non-healing ulcers, and gangrene of the diseased leg. CLI is associated with high rates of limb loss and mortality worldwide. It has been reported that about 25% of CLI patients require major amputation within 6 months [1]. The reported mortality for patients with rest pain is approximately 25% at 1 year and >50% at 5 years [1], resulting in a significant health burden.

Restoration of blood flow to the ischemic limbs is crucial to prevent tissue injury after arterial occlusion. Therapeutic angiogenesis

has emerged as a potential strategy for PAD patients to promote the growth of new vessels and thereby to supply sufficient blood flow to the ischemic limbs [2]. Angiogenesis, promoted by naked plasmid DNA or recombinant proteins, is a tightly regulated process that involves proteolytic digestion of the extracellular matrix (ECM), proliferation and invasion of vascular endothelial cells (ECs) and formation of functional capillaries [3]. Although angiogenic factors, such as vascular endothelial growth factor A (VEGF), hepatocyte growth factor and fibroblast growth factors, have provided encouraging results in the therapy of CLI, angiogenesis induced by these angiogenic factors are associated with vascular leakage, peripheral edema, non-reduction of amputation and non-improvement of peak walking time [4-6]. Therefore, new angiogenic factors should be identified and tested for angiogenic promotion in differentiated ECs to avoid these limitations.

## Survivin affects angiogenesis

As noted, therapeutic angiogenesis shares some endothelial growth factors and markers of angiogenesis with pathological angiogenesis [7], such as tumor angiogenesis. Survivin (SVV), a protein expressed in the embryonic tissues and malignancies, has been found to have highly pleiotropic functions and is involved in the regulation of tumor progression, metastasis and angiogenesis. SVV is a member of the family of inhibitor of apoptosis proteins (IAPs) and contains a single baculovirus IAP repeat domain that may interfere with vascular ECs apoptosis by interacting with caspase-3, 8, and 9. SVV has additional unique ability to promote cell survival in that it is essential for the mitosis and cell-cycle progression. The mechanism by which SVV enhances angiogenesis is likely related to its ability to inhibit ECs apoptosis, thereby enhancing cell survival [8]. In addition, previous investigations have shown that the expression of matrix metalloproteinase (MMPs) is up-regulated in the endometriosis and tumors in response to SVV expression [9, 10]. MMPs play a crucial role in the degradation of ECM and basement membrane. Therefore, SVV is proposed to contribute to the invasion of endometriotic cells and tumor cells.

In view of the unique angiogenic properties of SVV in tumors, adenovirus-mediated SVV gene was transfected into rat aortic ECs (RAECs) to test the hypothesis that overexpression of SVV is able to inhibit RAECs apoptosis, promote their proliferation, migration and invasion, and induce angiogenesis which provides a new option for the therapeutic angiogenesis.

### Materials and methods

#### *Animals, cell lines, and adenoviral vectors*

BALB/c nude mice were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). All animal procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of Chongqing Medical University. RAECs were purchased from the American Type Culture Collection (ATCC; Manassas, USA) and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

The recombinant adenoviral vectors (Ad) expressing murine SVV and fused with green fluorescent protein gene (GFP, Ad-GFP-SVV) were constructed by Life Technologies Inc. (California, USA). The recombinant Ad expressing the GFP (Ad-GFP) alone was used as a control.

#### *Adenovirus vector transfection*

Cells were seeded into 6-well plates (5×10<sup>4</sup> cells/well) and incubated overnight. When RAECs reached 50-60% confluence, the medium was removed and replaced with RPMI-1640 containing Ad-GFP (5 PFU/cell) or Ad-GFP-SVV (5 PFU/cell). Then, these cells were divided into three groups: RAEC (blank control group, untreated), Ad-GFP (negative control group, transfected by Ad-GFP) and Ad-GFP/SVV group (transfected by Ad-GFP-SVV). Cells were incubated at 37°C for 12 h and transfection was confirmed by viewing cells under a fluorescent microscope.

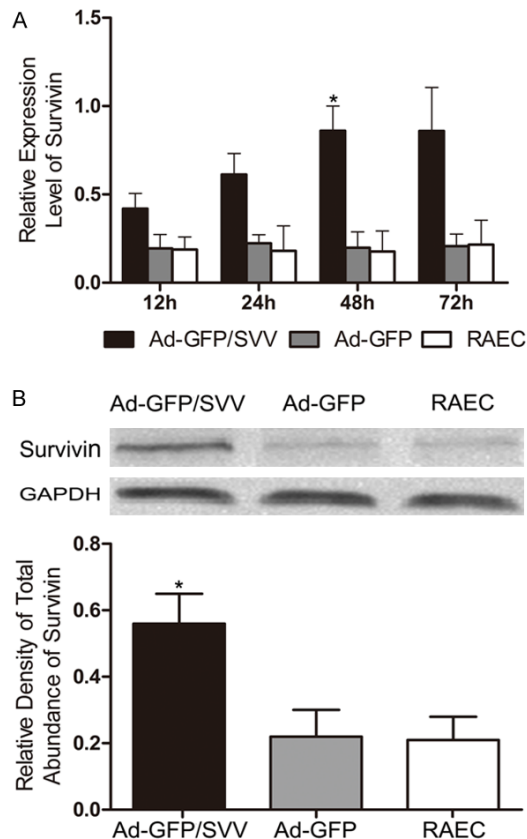
#### *Quantitative real-time RT-PCR (RT-qPCR)*

To detect the mRNA expression of SVV in RAECs, RT-qPCR was performed at 12 h, 24 h, 48 h, and 72 h after transfection. Total RNA was collected using Trizol reagent (Invitrogen, CA) following the manufacturer's instructions. The concentration and purity of total RNA were detected with the Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). PCR was carried out with cDNA derived from 50 ng of RNA, 1 unit of Taq polymerase and reaction kits at a final volume of 20 µl. Conditions for PCR were as follows: denaturation at 95°C for 15 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec. The relative expression of target gene was calculated with the comparative threshold cycle (Ct) method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences were as follows: 5'-TTTTGTGGCTT-TGCTCTATTGT-3' (sense), 5'-GGTAGGAGGACTC-ATCAGAAGGA-3' (antisense) for SVV; 5'-GCAAGTTCAACGGCACAG-3' (sense), 5'-GCCAGTAGA-CTCCACGACAT-3' (antisense) for GAPDH.

#### *Western blot assay*

The protein expressions of SVV, cellular cycle and apoptosis related proteins and MMPs in cells were detected by western blot assay. Cells were washed with cold phosphate buffer solu-

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**Figure 1.** mRNA (A) and protein (B) expressions of SVV in RAECs significantly increased at 48 h after SVV transfection (Ad-GFP/SVV) as compared to negative control group (Ad-GFP) and blank control group (RAECs, \* $P < 0.05$ ). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

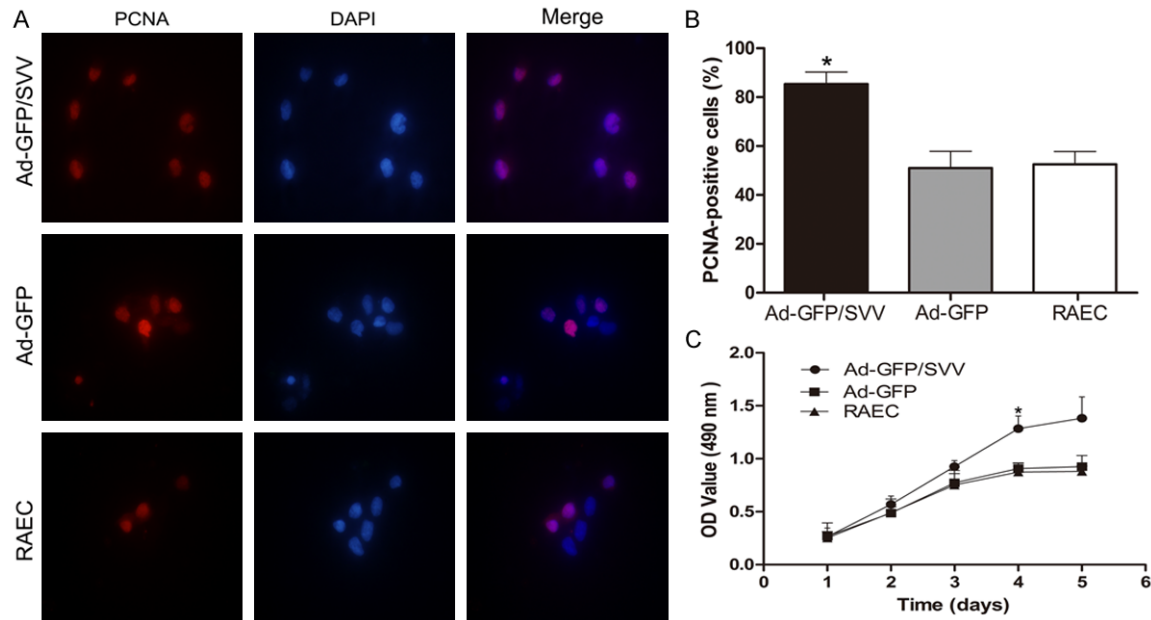
tion (PBS) and lysed in lysis buffer (pH 7.4, 150 mmol/l of NaCl, 50 mmol/l of Tris-HCl, 2 mmol/l of EDTA, 1% NP-40) for 30 min. The protein concentration was determined with Bradford dye-binding assay. GAPDH or  $\alpha$ -tubulin was used as an internal loading control. Total protein (20 mg/lane) was separated by 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was then blocked with primary polyclonal antibody at 4°C overnight. Afterward, the membranes were washed with tris buffer solution containing 0.05% Tween-20 and incubated with HRP-conjugated anti-mouse secondary antibody at 37°C for 1 h. After washing three times, band detection was conducted using enhanced chemiluminescence on a UVP gel imaging system (Upland, USA). The relative abundance of protein was quantified using the Quantity One software (Bio-Rad, Hercules, CA, USA).

### Cell viability/proliferation assay

Cell viability was measured via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, cells were seeded into 96-well plates at  $1 \times 10^4$  cells/well and maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. At the pre-designed time points, 20  $\mu$ L of MTT in Hank's balanced solution was added to each well at a final concentration of 5  $\mu$ g/mL followed by incubation at 37°C for 4 h. The medium was then removed and 200  $\mu$ L of dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance was measured at 490 nm using a microplate reader (Thermo Scientific, USA). Each experiment was repeated three times. As the proliferating cell nuclear antigen (PCNA) is highly correlated with cell proliferation, the immunofluorescence staining of PCNA was used to detect the proliferation of RAECs. RAECs were cultured on coverslips in plates and fixed in 4.0% paraformaldehyde in PBS (pH 7.4) for 15 min, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum albumin (BSA) for 1 h. The cells were then incubated for 2 h at 37°C with rabbit PCNA monoclonal antibody (1:200, Abcam, UK). Then, cells were treated with Cy3-conjugated goat-anti-rabbit IgG (1:100, Abcam, UK) and nuclei were counterstained with DAPI. Images were acquired at a magnification of  $\times 1000$  under a Nikon microscope (Nikon Inc., Japan).

### Cell migration and invasion assays

The 24-well transwell plate with 8- $\mu$ m polyethylene terephthalate membrane filters was applied to test cell migration. Cells ( $2 \times 10^5$  cells/ml) were seeded in the upper chamber with 200  $\mu$ L of serum-free medium, while 600  $\mu$ L of RPMI-1640 with 10% FBS was added to the bottom chamber. The chamber was incubated at 37°C in a humidified environment with 5% CO<sub>2</sub> for 24 h. Then, the non-migratory cells in the upper chamber were removed using a cotton swab, and cells in lower chamber were fixed in 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 25 min and counted in three random fields under an inverted microscope. The cell invasion assay was essentially similar to the transwell migration assay except for the membrane filter being coated with matrigel matrix (BD Biosciences, USA) prior to the assay.



**Figure 2.** Effect of SVV on the proliferation of RAECs. A. Immunofluorescence staining for PCNA in RAECs after adenovirus transfection (original magnification: 1000 $\times$ ). B. Percentage of PCNA-positive cells after transfection. C. MTT assay. Proliferation of cells in Ad-GFP/SVV group significantly increased as compared to two control groups (Ad-GFP and RAECs, \* $P < 0.05$ ). SVV significantly enhanced the proliferation of RAECs. PCNA, proliferating cell nuclear antigen.

#### Cell apoptosis assay

The apoptosis of RAECs was induced by addition of anti-Fas antibody (0.5 mg/mL) and detected by TdT-mediated dUTP nick end labeling (TUNEL) staining and flow cytometry. The *in situ* cell death fluorescence detection kit was used for TUNEL staining according to the manufacturer's instructions. DNA chromatin fragments were stained with DAPI. Apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation. The cells after anti-Fas antibody treatment were collected and suspended in 500  $\mu$ L of binding buffer, and then 5  $\mu$ L of Annexin-V-fluorescein isothiocyanate and 5  $\mu$ L of propidium iodide were added, followed by incubation for 15 min in dark. Flow cytometry (BD, Franklin Lakes, NJ, USA) was performed to detect apoptotic cells. Each experiment was repeated 3 times.

#### Tube formation assay

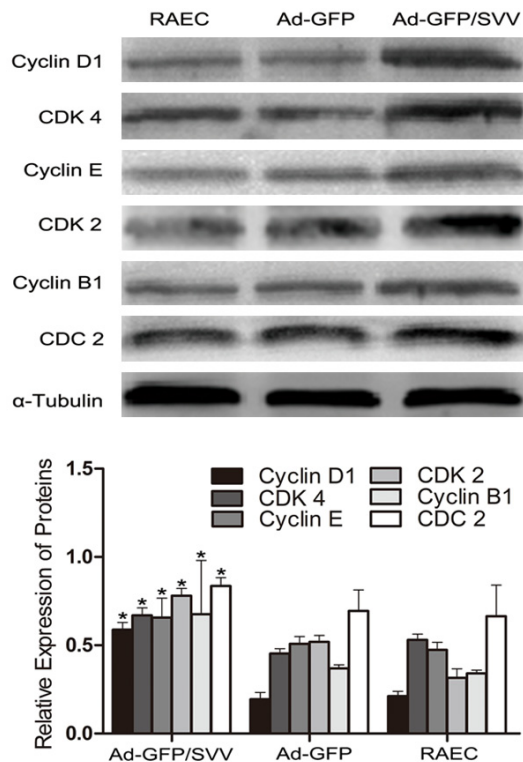
Tube formation assay *in vitro* was used to evaluate angiogenic activity of RAECs. 24-well plates were coated with 250  $\mu$ L of growth factor-reduced matrigel (BD, Biosciences, USA). RAECs ( $5 \times 10^4$ ) were suspended in 500  $\mu$ L of serum-free conditioned medium, then plated

onto the polymerized matrigel and incubated at 37°C for 6 h. The capillary tube like structures formed by RAECs were photographed under a phase contrast inverted microscope, and the tubes and branches were counted with the Image Pro Plus 6.0 (Media Cybernetics, Atlanta, USA). The supernatant was collected after tube formation assay, and VEGF content was detected by enzyme-linked immunosorbent assay (R&D, USA) according to the manufacturer's instructions. The experiment was repeated thrice.

#### *In vivo* matrigel plug assay

Six-week-old nude mice were divided into three groups with four animals (2 males and 2 females) in each group. Mice were injected subcutaneously injection of matrigel in Ad-transfected RAECs (250  $\mu$ L of matrigel plus 250  $\mu$ L of serum-free medium containing  $2 \times 10^6$  cells) at the abdomen. Seven days later, mice were sacrificed and the Matrigel plugs were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Then, 5  $\mu$ m-thick sections were obtained and subjected to hematoxylin and eosin (H&E) staining, Masson's trichrome staining and CD31 staining. The blood vessel formation in matrigel plugs and cap-





**Figure 3.** Expressions of cell cycle related proteins in RAECs after transfection. Results showed the expressions of cyclin D1, cyclin E, cyclin B, CDK 4, CDK 2 and CDC 2 increased significantly in Ad-GFP/SVV group when compared with two control groups (Ad-GFP and RAECs, \* $P < 0.05$ ).

sules were quantified by three investigators blind to this study at a high magnification, and average was calculated.

#### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). The analysis of variance was used for comparisons among three groups, and a two-tailed Student t-test was used for comparisons between two groups. A value of  $P < 0.05$  was considered statistically. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

#### Results

##### SVV expression in RAECs was up-regulated after Ad-GFP-SVV transfection

qRT-PCR was performed to detect the SVV mRNA expression in RAECs at 12 h, 24 h, 48 h, and 72 h after transfection with Ad-GFP-SVV. No SVV expression was found in blank and negative control groups. There were no significant

difference in SVV mRNA expression in Ad-GFP-SVV group between 48 h and 72 h. Therefore, 48 h was determined as the optimal transfection time. Then, western blot assay was applied to detect the SVV protein expression in RAECs at 48 h. Results showed that SVV protein expression in Ad-GFP/SVV group was significantly enhanced (**Figure 1**).

##### SVV enhanced RAECs proliferation

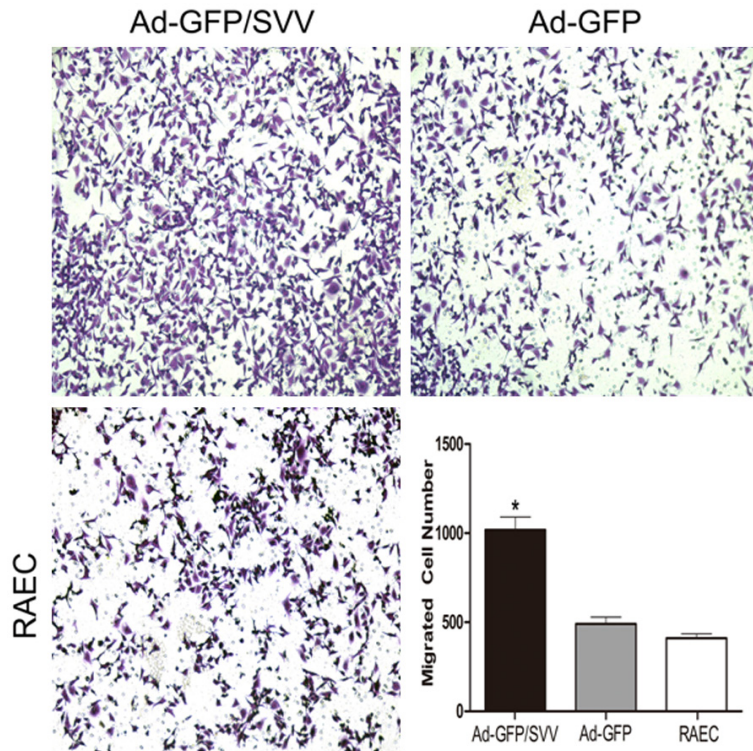
PCNA is a marker of cell proliferation. The PCNA expression was detected by immunofluorescence staining. As shown in (**Figure 2A** and **2B**), SVV over-expression in RAECs significantly increased PCNA expression. Then, the effect of SVV on cell growth was explored by MTT assay. As shown in (**Figure 2C**), SVV over-expression significantly enhanced the growth of RAECs, but there were no marked difference between blank control group and negative control group in cell growth. Meanwhile, western blot assay indicated that the protein expressions of cyclins (cyclin B1, cyclin D1, and cyclin E) and CKDs (CDC2, CDK 4 and CDK2) were significantly increased in Ad-GFP/SVV group (**Figure 3**). These findings suggest that SVV significantly elevates the proliferation of RAECs by promoting cell cycle progression.

##### SVV promotes RAECs invasion and migration

The effect of SVV on the RAECs migration was examined by transwell assay. The number of cells migrating through the membrane in Ad-GFP/SVV group significantly increased as compared to negative and blank control groups (**Figure 4**). These indicate that SVV significantly enhances the migration of RAECs. To investigate the effect of SVV on the RAECs invasion, a matrigel chamber assay was carried out. Results showed cells with SVV over-expression degraded the matrigel basement membrane matrix and the number of cells migrating through the membrane significantly increased in Ad-GFP/SVV group as compared to remaining groups. Moreover, the transient transfection of SVV significantly increased the expressions of MMP-2, 7 and 9 in AD-GFP/SVV group when compared with two control groups (**Figure 5**).

##### SVV inhibits RACEs apoptosis by suppressing caspases

TUNEL- and DAPI-positive RAECs after SVV transfection are shown in (**Figure 6**). The



**Figure 4.** Transwell cell migration assay in RAECs infected with SVV and GFP (original magnification: 200×). The number of cells migrating through the membrane in Ad-GFP/SVV group significantly increased when compared with two control groups (Ad-GFP and RAECs, \* $P < 0.05$ ).

amount of TUNEL- and DAPI-positive RAECs was significantly decreased as compared two control groups. As shown in (Figure 7, 5.48% of RAECs became apoptotic after addition of anti-Fas antibody in Ad-GFP/SVV group. In negative and blank control groups, the proportions of apoptotic RAECs were 19.3% and 20.74%, respectively ( $P < 0.05$  vs Ad-GFP/SVV group). Furthermore, the expressions of apoptosis related proteins (cleaved-caspase-3, 8 and 9) were detected via immunohistochemistry (Figure 8). As expected, the expressions of cleaved-caspase -3, 8 and 9 significantly decreased in RAECs of Ad-GFP/SVV group as compared to two control groups. Altogether, these indicate that SVV protects against RAECs apoptosis induced by anti-Fas antibody.

#### SVV enhances tube formation in RAECs

SVV plays a key role in the tumor angiogenesis, and therefore, matrigel-coated tube formation assay was performed in RAECs after SVV transfection. After SVV transfection, more

tubes and branches were formed as compared to cells in control groups. Moreover, the VEGF expression in Ad-GFP/SVV group was significantly higher than in two control groups. These suggest that SVV contributes to an angiogenic environment (Figure 9).

#### SVV promotes angiogenesis in vivo

To determine whether SVV affects angiogenesis *in vivo*, matrigel plug assay was performed in 12 nude mice. As shown in Figure 10A, the plugs were harvested for H&E staining, Masson's trichrome staining and CD31 staining and the angiogenesis was evaluated. Results showed that SVV transfection significantly increased the number of neovessels in the plugs and capsules as compared to control groups (Figure 10B). These indicate that SVV, a novel

apoptosis inhibitor, exhibits potent angiogenic promotion.

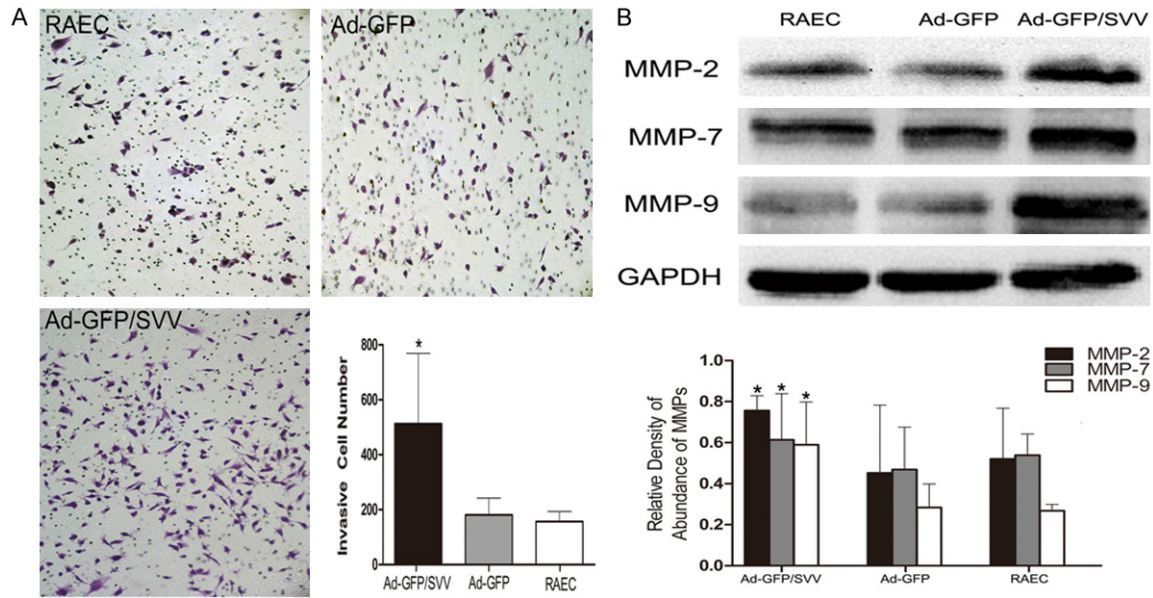
#### Discussion

One of the cornerstone requirements for angiogenesis is the continuous preservation of endothelial viability, a multifaceted process that involves the regulation of cell division and expression of specialized anti-apoptotic genes [11]. One such target gene is SVV which is an inhibitor of apoptosis, can promote cell proliferation, and is associated with the angiogenesis in tumors [12]. In present study, our results demonstrated that SVV over-expression could enhance the proliferation, migration, invasion, and apoptosis resistance of RAECs. Moreover, SVV activated the expressions of angiogenesis-related molecules in RAECs and robustly promoted angiogenesis *in vitro* and *in vivo*. All these findings suggest that SVV can be used to promote the angiogenesis in PAD.

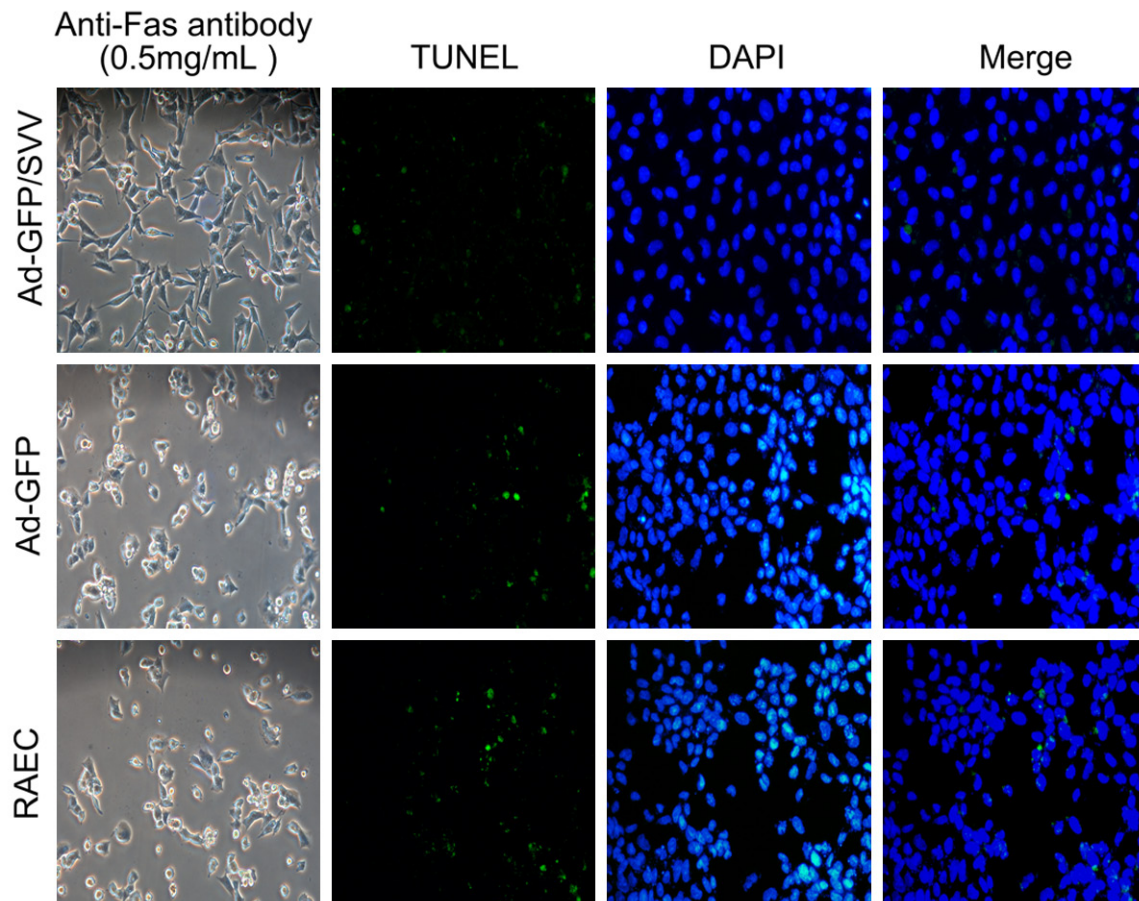
Cellular division is ultimately dependent on the progression of cell cycle, in which cells transit



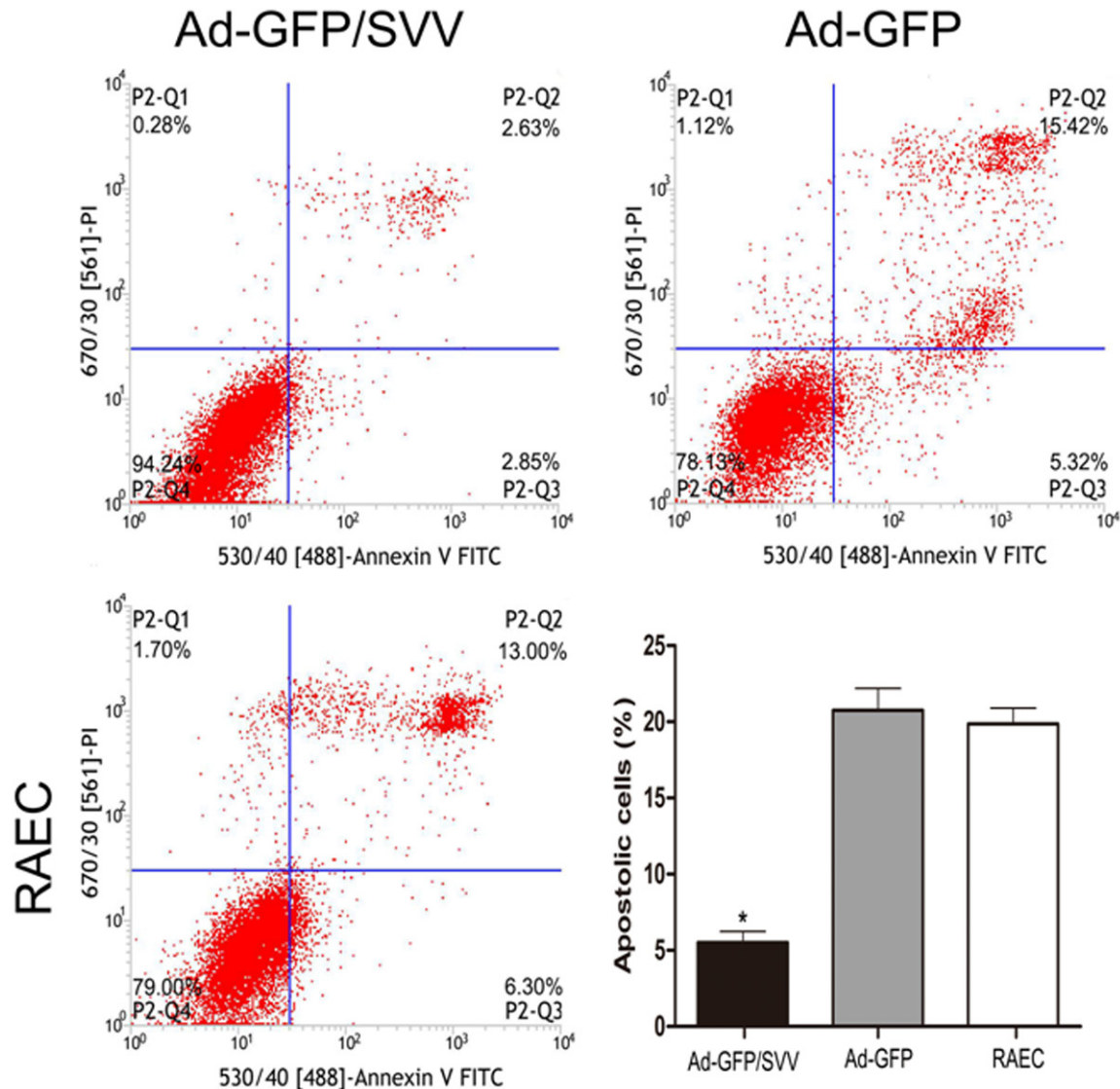
## Survivin affects angiogenesis



**Figure 5.** Matrigel cell invasion assay (A. Original magnification: 200×) and Western blot assay of MMPs expression (B) in RAECs after transient transfection with SVV and GFP. Results showed the number of invasive cells and the expressions of MMP-2, 7, and 9 increased dramatically in Ad-GFP/SVV group (\* $P < 0.05$ ). MMPs, matrix metalloproteinases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



**Figure 6.** Detection of apoptotic cells after anti-Fas antibody treatment via TUNEL staining and DAPI staining (original magnification: 200×). Representative photomicrographs of TUNEL staining showed apoptotic cells after anti-Fas antibody treatment in RAECs undergoing SVV transfection. SVV transfection effectively inhibited the apoptosis of RAECs, as evidenced by few TUNEL-positive cells.



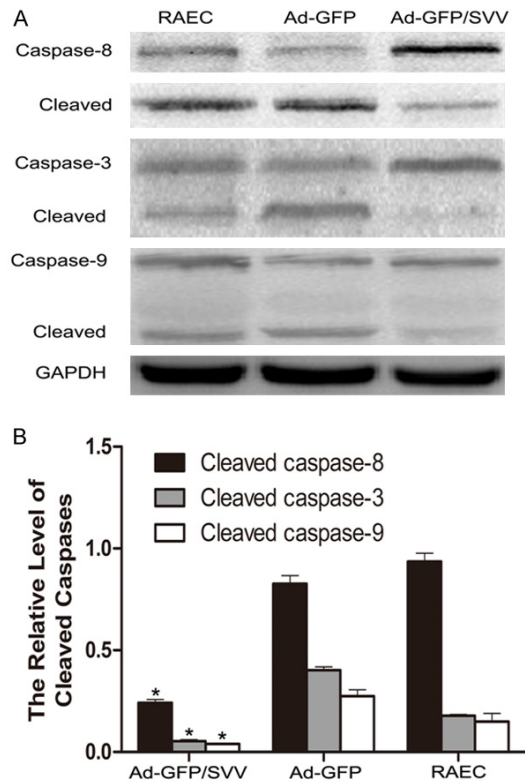
**Figure 7.** Detection of apoptotic cells after anti-Fas antibody treatment via flow cytometry. Cells in the upper right and lower right quadrants were early apoptotic cells and late apoptotic cells, respectively. SVV over-expression effectively inhibited the apoptosis of RAECs (\* $P < 0.05$ ).

through the G0/G1 phase to the S-phase, and eventually to the G2-M phase [13]. The crucial role of SVV in cellular division is unanimously accepted. In cell division, SVV acts as a regulator of polymerization and movement of spindle microtubules and cytokinesis in the metaphase and anaphase of mitosis [14-16]. Moreover, earlier studies also indicate that SVV

expression increases during G1 phase, and reaches a peak in G2/M phase, suggesting that it affects cell cycle progression [17]. In the present study, RAECs were successfully transfected with SVV gene, demonstrated by the elevated expressions of SVV at mRNA and protein levels. MTT assay indicated SVV over-expression promoted cell viability, which attrib-



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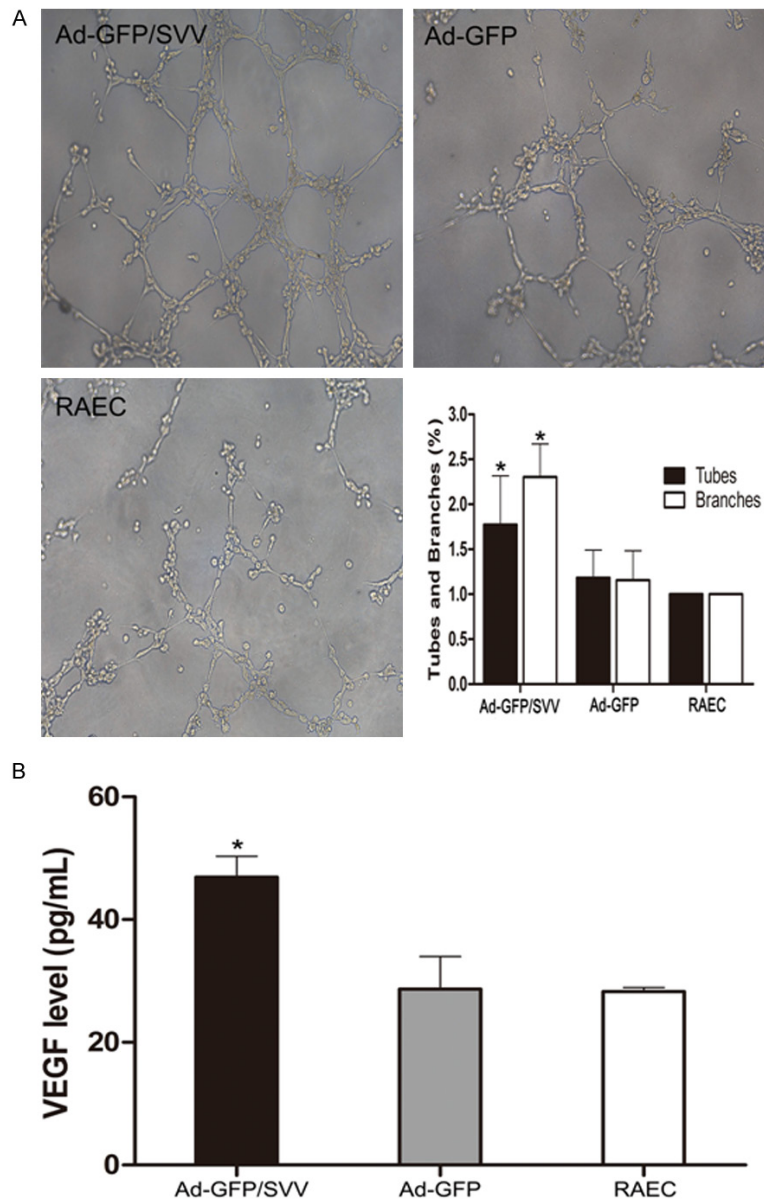
**Figure 8.** The expressions of apoptosis proteins in RAECs. A. The represented protein bands from Western blot assay. B. The quantification of protein bands. The protein expressions of cleaved-caspase-8, 3, and 9 decreased significantly in Ad-GFP/SVV group as compared to two control groups (Ad-GFP and RAECs, \* $P < 0.05$ ). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

uted to the expressions of cell cycle-related proteins. The cell cycle is regulated by a complex network of cyclins and cyclin-dependent kinases (CDKs), whose formation and activation are able to promote cell cycle progression. The cyclin-CDK complexes, cyclin-D1/CDK4 and cyclin-E/CDK2 control the G1 to S transition through phosphorylating the retinoblastoma protein and increasing PCNA expression, while cyclin B1 and Cdc2 regulate the progression into G2/M phase [18]. In present study, the expressions of cyclins (cyclin B1, cyclin D1, and cyclin E) and CDKs (CDC2, CDK 4 and CDK2) were significantly increased after SVV transfection. Moreover, the expression of PCNA, a subunit of DNA polymerase and a biomarker for the evaluation of proliferative activity of cells, was increased significantly in SVV transfection group. These were consistent with previous findings indicating that SVV expres-

sion is associated with cell proliferation reported in embryonic tissues and cancers [19, 20].

Ischemic diseases are the consequence of endothelial damage, and the main causes of this damage are inflammation and oxidative stress [21, 22], which have been reported to impair endothelial growth and angiogenesis, leading to cell apoptosis [23]. Therefore, to prevent ECs from apoptosis is crucial for the angiogenesis [7]. Apoptosis is regulated by a family of cysteine proteases termed caspases. The caspase family members can be divided into initiator (such as caspase-8, and 10) and effector (such as caspase-3, and 9) caspases. SVV is known as an inhibitor of caspases and able to block cell apoptosis. In Fas/Fas ligand (FasL) apoptotic pathway, binding of FasL to Fas results in the activation of caspase-8 and 10, ultimately leading to the activation of caspase-3 and 9. In our study, anti-Fas antibody was used to induce the apoptosis of RAECs, and the expressions of caspases in Fas/FasL death pathway were detected by Western blot assay. Results showed the number of apoptotic cells in RAECs after SVV transfection was significantly lower than in two control groups, as shown by flow cytometry and TUNEL staining. Furthermore, the expressions of cleaved-caspases 8, 3 and 9 were significantly suppressed in RAECs after SVV transfection. The suppression of caspases activity in SVV over-expressing RAECs shown in the present study was consistent with the general function of IAP proteins as caspase inhibitors in tumors.

Migration and invasion of vascular ECs are essential events in the process of angiogenesis. Vascular ECs need to loose their mutual contact and interact with basement membrane. Various proteins (such as MMPs and VEGF) influence angiogenesis by destabilizing the vascular structure and degrading the extracellular matrix, thus favoring EC sprout from pre-existing capillaries. The mutual regulation in expressions of VEGF and MMPs accelerate the proliferation and migration of ECs in the angiogenesis [24]. In addition, previous studies have indicated that the SVV induced cell migration has involvement of the formation of SVV-XIAP (X-linked IAP) complex, which may promote the nuclear factor- $\kappa$ B-dependent activation of a process that promotes cell motility, including enhanced production and deposition of fibro-



**Figure 9.** Effects of SVV on tube formation and VEGF expression in RAECs. A. Representative photographs from tube formation assay (original magnification: 200×). The mean number of tubes and branches in Ad-GFP/SVV group significantly increased when compared with control groups (Ad-GFP and RAECs, \* $P < 0.05$ ). B. Enzyme-linked immunosorbent assay for VEGF in the cell supernatant. VEGF content in Ad-GFP/SVV group was markedly higher than in control groups (Ad-GFP and RAECs, \* $P < 0.05$ ).

nectin [25, 26]. In the present study, transwell assay and matrigel chamber assay were employed to investigate the migration and invasion of ECs after SVV transfection, respectively. The numbers of migrating and invasive cells significantly increased in SVV transfection group as compared to two control groups. Meanwhile, the expressions of MMP-2, 7, and

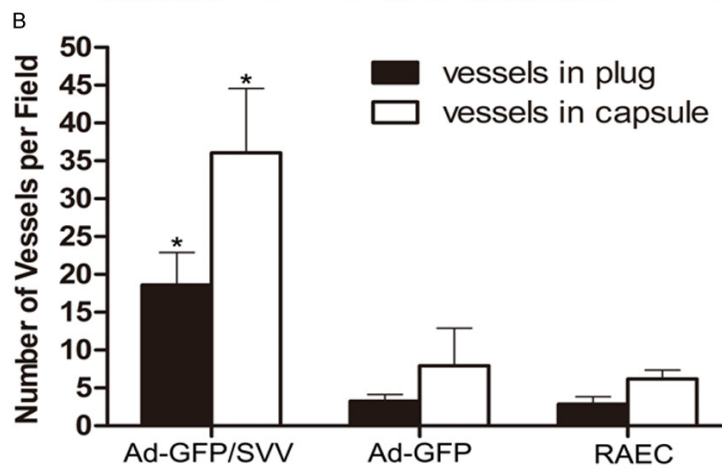
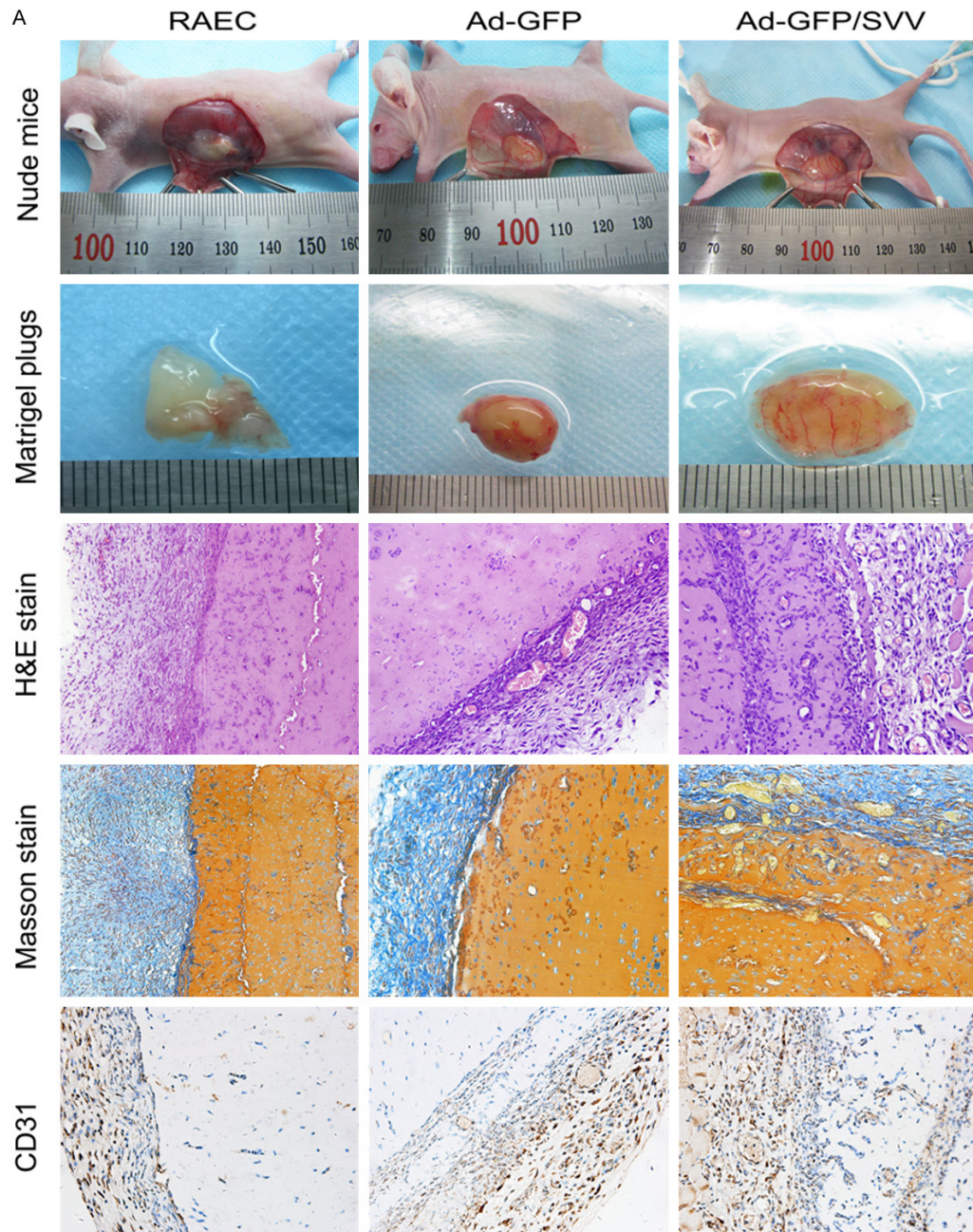
9 in these cells were significantly enhanced. The matrigel matrix is a solubilized basement membrane preparation extracted from the mouse sarcoma, which is composed of laminin, heparin sulfate proteoglycans, and collagen IV etc [27]. MMP-7 participates in the degradation of ECM components, such as elastin, type IV collagen, proteoglycans, fibronectin, vitronectin and aggrecan [28]; MMP-2 is also known as '72 kD type IV collagenase' and MMP-9 as '92 kD type IV collagenase' [29]. Our results demonstrated that SVV transfection promoted the invasion of RAECs by increasing MMPs expression.

Angiogenesis occurs in stages, which orchestrate a network of cooperative interactions. To confirm the angiogenesis capacity of SVV *in vitro* and *in vivo*, tube formation assay and matrigel plug assay were used in the present study. Results showed the capillary-like tubes *in vitro* and the neovessels in plugs and capsules *in vivo* in SVV group significantly increased, which may be attributed to two reasons: first, SVV transfected cells were incorporated into pre-existing capillaries. Our results showed that SVV promoted RAECs proliferation and invasion *in vitro*, and further to increase the formation of capillary-like tube in

the matrigel basement membrane. Thus, the cells may directly participate in angiogenesis, and SVV transfection promotes the angiogenic effect. Second, the soluble factors in local microenvironment, such as VEGF, may enhance the angiogenic capacity of ECs in pre-existing capillaries. Our results demonstrated that SVV over-expression could increase the VEGF ex-



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**Figure 10.** Effect of SVV on the angiogenesis of RAECs *in vivo*. A. Nude mice were subcutaneously injected with matrigel mixed with RAECs after transfection. Seven days later, animals were sacrificed and the plugs were collected and photographed. Sections of matrigel plugs were processed for H&E staining, Masson's trichrome staining and CD31 staining (original magnification: 200×). B. Quantification of angiogenesis in the matrigel plugs and capsules. The number of vessels in the plugs and capsules significantly increased in Ad-GFP/SVV group as compared to two control groups (Ad-GFP and RAECs, \* $P < 0.05$ ).

pression in local microenvironment. Meanwhile, the capillaries density in matrigel plug capsule originating from subcutaneous blood vessels of nude mice significantly increased in SVV group. This suggests that SVV induced angiogenesis *in vitro* and *in vivo* may occur simultaneously through a paracrine dependent manner. This conclusion is consistent with previous findings that SVV may regulate angiogenesis not only via controlling ECs proliferation, but via increasing secretion of VEGF as in tumor cells [30-32].

## Conclusions

Collectively, our study demonstrates that SVV can promote angiogenesis *in vivo* by enhancing the proliferation, migration, invasion, and apoptosis resistance of vascular ECs, which suggests the potential of SVV as a therapeutic intervention for PAD. However, there are several limitations in the present study. Previous investigations have shown that an enhanced SVV protein expression was detectable in granulation tissues and participated in the angiogenesis by increasing ECs viability [33]. Moreover, recent studies also define a regulatory role of survivin in normal adult cells, such as polymer-phosphonuclear cells, hematopoietic cells [34-36]. These findings in normal tissues indicate that SVV expression is not tumor specific, and the tumorigenicity of SVV should be further investigate to ensure the safety. Furthermore, animal PAD models should be established to investigate the angiogenic capacity of SVV in the ischemic environment, because the roles of apoptosis, proliferation, migration and invasion of vascular ECs in such environment are different from those in normal tissues with good blood perfusion. These limitations will be resolved in our future studies.

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## Disclosure of conflict of interest

None.

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