

# Effects of adipose-derived stromal cells and endothelial progenitor cells on adipose transplant survival and angiogenesis

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## Research

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# Abstract

**Background:** A paracrine mechanism is thought to mediate the proangiogenic capacity of adipose-derived stromal/stem cells (ASCs). However, the precise mechanism by which ASCs promote the formation of blood vessels by endothelial progenitor cells (EPCs) is unclear.

**Methods:** We cocultured ASCs with EPCs at various concentrations to study the effects on angiogenesis. The supernatant from cultured ASCs was cocultured with EPCs to evaluate the effects on the expression of vascular endothelial markers in EPCs, as well as capacity for migration and invasion. We then mixed ASCs with EPCs and transplanted them with adipose tissue into New Zealand white rabbits to evaluate the effects on angiogenesis in adipose tissue grafts.

**Results:** As the relative abundance of ASCs cocultured with EPCs increased, the rate of angiogenesis among EPCs decreased. The supernatant from ASC cultures increased the migration and invasion of EPCs and upregulated the expression of vascular endothelial markers in EPCs. *In vivo*, ASCs promoted the production of blood vessels by EPCs.

**Conclusions:** ASCs are not recruited as structural components of blood vessels but do appear to regulate endothelial progenitor-mediated angiogenesis. The results obtained show that ASC paracrine signaling promotes the formation of blood vessels by EPCs. ASC paracrine signaling appears to promote angiogenesis by increasing the migration and invasion of EPCs and simultaneously upregulating the expression of vascular endothelial markers in EPCs. The results of *in vivo* experiments showed that ASCs promote the formation of blood vessels in EPC cultures.

## Background

Autologous fat transplantation technology was first introduced more than 100 years ago. Due to its unique advantages and remarkable clinical effects, it has attracted increasing attention from experts in the field of plastic surgery. Autologous fat transplantation has numerous advantages such as the availability of source material, decreased risk for rejection, a natural feel, minimal trauma, reproducible results, and easy removal. The major obstacle currently faced by autologous fat transplantation is autologous absorption. Most cases of autologous absorption occur in the first 3 months after transplantation, with the rate of absorption reaching 50%. The main reason for autologous absorption is the inability to expediently establish an effective blood supply to the fat graft. It is believed that distances > 2 mm between fat granules and the arterial blood vessels will result in insufficient nutrient supply to the transplanted fat. Portions of the fat will become necrotic, liquefy, and become absorbed [1]. Therefore, it is very important to establish a new blood supply that can provide sufficient nutrients for transplanted fat soon after transplantation.

In recent years, numerous studies have focused on the stem cells derived from fat. It has been shown that adipose-derived stem cells (ASCs) can promote the survival of autologous fat transplantation. Yuan *et al.* demonstrated that the addition of exogenous ASCs during transplantation significantly increased the

retention rate and the ratio of vascularized adipose tissue throughout the graft. Meanwhile, expression levels of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were higher in adipose grafts cotransplanted with ASCs [2].

The main mechanism of vascular repair in ischemic tissue requires the recruitment of endothelial progenitor cells (EPCs) in circulating blood. When the body undergoes pathological changes such as trauma and ischemia, EPCs migrate and colonize the ischemic site, secrete pro-angiogenic factors, and promote the growth of collateral vessels to ischemic tissue. EPCs are also known to participate in the organization of angiogenesis and to promote tissue repair[3].

We therefore cocultured ASCs and EPCs in order to observe the effects on EPC tube formation, migration, and invasion. This study aimed to investigate whether ASCs improve the survival of fat grafts by promoting EPC-mediated angiogenesis.

## Materials And Methods

### *Isolation and characterization of peripheral blood EPCs (PB-EPCs)*

For the isolation of EPCs, peripheral blood (10 ml/kg) was obtained from New Zealand white rabbits by puncturing the ear vein. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Ficoll-Plaque Plus (GE Healthcare, America). Mononuclear cells were then washed and plated on six-well plates that were coated with human fibronectin (Sigma, America) at a concentration of 3  $\mu\text{g}/\text{cm}^2$  or rat tail collagen I (BD, America) at a concentration of 10  $\text{mg}/\text{cm}^2$ . Cells were supplemented with EGM-2 MV BulletKit medium (Lonza Corp, Switzerland). Mononuclear cells were incubated at 37°C, 5% CO<sub>2</sub>, and fed daily with EGM-2. At 3 to 7 days post-plating, adherent EPC were detached with 0.025% trypsinase containing 0.02% EDTA. The same procedure was performed for the subsequent 3 passages (approximately 1 week of culture). All experiments were performed with cells that were cryopreserved at passage 3.

EPCs isolated from rabbits were characterized based on their ability to take up Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) and bind to *Ulex europaeus* agglutinin-1 (UEA-1), as previously described. Briefly, P3 cultures of EPCs were seeded on 6-well plates and incubated with 20  $\mu\text{g}/\text{ml}$  Alexa Fluor 488-labeled Dil-Ac-LDL (Molecular Probes) for 4 h or with 15  $\mu\text{g}/\text{ml}$  rhodamine-labeled UEA-1 (Vector) for 1 hour. After incubation, cells were washed with serum-free medium and detected under fluorescence microscopy. Immunostaining for vascular endothelial growth factor receptor 2 (VEGFR2) and CD31 was performed as previously described. Briefly, EPC cultures were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 minutes, then blocked with 5% bovine serum albumin (BSA) at 37°C for 1 hour. Cells were then incubated with mouse anti-mCherry (1:1000; Biolegend) at 4°C overnight. Sections were washed 4 times in PBS and stained for 2 h in the dark at room temperature with Alexa Fluor 488 goat anti-mouse antibody(1:1000; Molecular Probe). After 4 washes with PBS, the slides were mounted in medium containing DAPI (Vectorshield).

### *Isolation and culturing of adipose stromal cells*

Adipose tissue was obtained from the thighs and groins of New Zealand white rabbits and cut into 2-mm pieces. Tissues were then washed twice with PBS containing 2% penicillin/streptomycin and once with DMEM/F12 medium. The cleaned fat blocks were transferred into a 15-ml centrifuge tube and agitated in 1 mg/ml Liberase TL solution prepared in DMEM/F12 medium (Invitrogen) supplemented with 5% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin, for 1 h at 37°C and 180 rpm. Single cells were obtained by filtering the solution through a 70-µm cell strainer. Samples were then centrifuged at 1000 rpm for 5 min to separate the stromal cell fraction from the adipocytes. The pellet was treated with red blood cell (RBC) lysis buffer to eliminate erythrocytes, then resuspended in EGM-2MV media. The ASC monolayers were split upon achieving 80% confluence and used at passage 3.

### *Molecular phenotyping of ASCs and EPCs cultured in vitro*

Quantitative real-time PCR (qRT-PCR) was performed to measure gene expression in EPCs and ASCs cultured *in vitro*. ASCs and EPCs were seeded into 6-well plates. Total RNA was extracted from cultured cells and fresh isolated PBMC with Trizol reagent (Invitrogen), then converted into cDNA with a First Strand cDNA Synthesis kit (Thermo Scientific). QRT-PCR was conducted using SYBR Green I Master mix (Applied Biosystems) with an ABI 7500 instrument. The primers for CD31, CD34, CD45, VEGF, VEGFR2, and ANGPT1 (Angiopoietin-1) are summarized in Table 1. Relative gene expression was normalized to PBMC levels of GAPDH expression. All experiments were performed in triplicate. The results are expressed as mean±SEM.

### *Two-dimensional assessment of Matrigel angiogenesis*

To test the ability of endothelial progenitor cells-mesenchymal stem cell (EPC-MSC) cocultures to form tubes *in vitro*, EPCs and ASCs were resuspended in EGM-2 basal medium (10% heat-inactivated FBS, without growth factors), then seeded at cell number ratios of 100:0, 80:20, 67:33, 50:50, 33:67, 20:80, and 0:100 onto 24-well plates precoated with thick Matrix gel (300 µl/well, Matrigel matrix growth factors reduced, BD). The final density of cells was 10<sup>5</sup> cells/ml/well. The plates were incubated for 6 h at 37°C and 5% CO<sub>2</sub>. Tube formation was observed with inverted phase-contrast microscopy. For each sample, a total of 6 pictures were randomly captured from three wells in parallel. Tube length and number of branches were measured with Image-Pro Plus software. The results are expressed as mean±SEM.

To analyze the pro-angiogenic activity of ASCs, ASCs supernatant was collected after 48 h of cultivation and subjected to a two-dimensional angiogenesis assay. EPC supernatant was used as the internal control. EGM-2 basal medium and EGM-2 MV complete medium were used as culture controls. As described previously, 10<sup>5</sup> EPCs were resuspended in 1 ml of ASC supernatant, EPC supernatant, EGM-2 basal medium, or EGM-2MV complete medium, then seeded into 24-well plates precoated with thick Matrix gel. Tube formation was analyzed with the methods described above.

### *Coculture of ASCs and EPCs*

The expression of angiogenesis markers in EPCs cocultured with ASCs was examined with qRT-PCR. EPCs were resuspended in EGM-2 basal medium, then seeded in a total volume of 2.7 ml containing  $5.4 \times 10^5$  cells in 6-well plates. A total of  $3 \times 10^5$  ASCs that had been resuspended in 1.5 ml EGM-2 basal medium were added to each transwell insert (pore size 2  $\mu\text{m}$ , Corning). The same volume of EGM-2 basal medium or EGM-2 MV complete medium was added to a different transwell insert as a control. After coculture for 24 h, the EPCs in the lower chamber were harvested for total RNA extraction with Trizol reagent (Invitrogen). RNA (2  $\mu\text{g}$ ) was reverse-transcribed using the First Strand cDNA Synthesis kit (Thermo scientific). QRT-PCR was conducted using SYBR Green I MasterMix (Applied Biosystems) with an ABI 7500 instrument. The primers for VEGF, angiopoietin-1/2, Flt-1, KDR, Tie-1/2, VE-cadherin, CD31, and GAPDH are shown in Table 1. Experiments were performed in triplicate for each gene. The results are expressed as mean $\pm$ SEM.

### *EPC transmigration and invasion*

The transwell migration assay was used to investigate the chemotactic ability of ASCs cocultured with EPCs. Briefly, 500  $\mu\text{l}$  EPCs ( $5 \times 10^4$  cells) diluted with EGM-2 basal medium were seeded into the cell culture inserts (pore size 8  $\mu\text{m}$ , Corning) in the 24-well plate. Then, 750  $\mu\text{l}$  of suspended ASCs ( $7.5 \times 10^4$  cells) or the same volume of control medium (EGM-2 basal medium or EGM-2 MV complete medium) was added to the outer compartment of each well of the 24-well plate. After 8 h of coculture, the culture inserts were carefully removed and fixed in 4% paraformaldehyde. Non-migrated cells on the upper side of the membrane were removed with a cotton swab. The cells that had migrated were stained with crystal violet.

Invasion assays were used to analyze the proteolytic activity and capacity for migration of EPCs cocultured with ASCs. As described above, the EPCs were seeded into BioCoat matrigel-pretreated chambers (pore size 8  $\mu\text{m}$ , Corning). ASCs were cocultured in the outer compartment. Twenty-four hours later, cells that had migrated to the bottom side of the membrane were fixed and stained with crystal violet. All the experiments were performed in triplicate. Cell counts for transmigration and invasion assays were obtained with Image-Pro Plus software and expressed as mean $\pm$ SEM.

### *Seeding of an adipose tissue implant with ASCs and EPCs*

To evaluate the capacity of ASCs to promote vascularization among EPCs in the setting of fat implantation, implanted adipose tissue was seeded with ASCs and EPCs.

Donor fat was obtained from New Zealand white rabbits that had been anesthetized with pentobarbital (0.01 ml/g body weight at 5 mg/ml in 5% ethanol/PBS, intraperitoneally). The fat tissue obtained was cut into small cubes (approximately 5 mm $\times$ 5 mm $\times$ 3 mm) and kept in EGM-2 basal medium in a 24-well plate. To study fat transplant angiogenesis *in vivo*, fat tissue was embedded in polymerized Matrigel, then mixed with EPCs or EPCs+ASCs and implanted subcutaneously in C57BL/6 nude mice (20 g; Beijing Vital River Laboratory Animal Technology). A total of  $4 \times 10^5$  EPCs or EPCs+ASCs (ratio 1:1) were resuspended in 200  $\mu\text{l}$  matrigel, then added to the fat tissue. The tissue and cells that had been embedded in gel were

incubated for 30 minutes at 37°C to allow for solidification. Incisions of 4 mm in length were made on the backs of anesthetized nude mice, and the gel-entrapped tissue was implanted subcutaneously. Thirty days after implantation, mice were sacrificed via gas inhalation (CO<sub>2</sub>). Fat tissue was harvested and subjected to immunohistochemistry to evaluate vascular network formation. All experiments were performed at Kunming Medical University in compliance with the university's guidelines for the ethical treatment of experimental animals.

### *Statistical analysis*

All data are presented as means±SEM. SPSS11 software was used for statistical analysis. The independent-sample t-test was used for comparisons between groups. Results with  $p \leq 0.05$  were considered to be statistically significant.

## **Results**

### ***Characterization of EPCs and ASCs isolated from rabbit***

Generally, 4-7 days after cultivation, sparse adherent cells began to appear in the primary EPC cultures. These adherent cells displayed a typical cobblestone-like morphology on fibronectin or collagen I-precoated plates. After 9-12 days, the rapidly proliferating EPCs formed several monolayer colonies with cobblestone appearance. These colonies were designated as passage 1 (P1). The P3 EPCs showed relatively homogeneous spindle-like morphology under inverted phase-contrast microscopy (Fig. 1A). The P3 EPCs had functional characteristics of classical EPC cultures, such as the ability to take up Ac-LDL and bind to UEA-1 (Fig. 1B). The P3 EPCs also stained positively for CD31, an endothelial cell marker (Fig. 1C).

ASCs were isolated from rabbit adipose tissue through collagenase digestion. Twenty-four hours after seeding of the stromal vascular fraction (VSF) pellet, most cells were found adhering to the surface of the culture plates. These cells were referred to as ASCs. As shown in Fig. 1D, the P3 ASCs cultured in complete EGM-2 medium displayed a fibroblast-like morphology. The results of *in vitro* differentiation assays showed that ASCs exhibited adipogenic and osteogenic potential (Fig. 1D).

### ***Molecular phenotypes of EPCs and ASCs***

As previously reported, both fibronectin and collagen I may be used as attachment factors in the isolation of EPCs. However, the molecular phenotypes of EPCs isolated with different approaches have not yet been compared. As shown in Fig 2, the two types of EPCs shared a common pattern of marker gene expression. Both expressed similar levels of common endothelial markers CD31 and vWF, but not VEGFR2. Both types of EPCs expressed VEGFR1 and hematopoietic stem cell marker CD34. In contrast, ASCs expressed higher levels of angiogenic factors (VEGFA and angiopoietin 1) but did not express CD31, vWF, CD34, CD45, VEGFR1, or VEGFR2. The gene expression pattern of ASCs indicated angiogenic potential.

### ***Tube formation in EPC and ASC cocultures in vitro***

ASCs have previously been described as multipotent cells with pericytic properties that stabilize endothelial network formation *in vitro* and *in vivo*. Here we tested whether ASCs could promote the formation of vessel-like structures through direct cooperation with EPCs to coassemble vessels in a 2D *in vitro* model. As shown in Fig. 3, EPC-ASC cocultures of varying ratios on matrigel did not generate well-defined capillary-like structures. Primitive networks composed of interrupted rings and agglomerates were found in cocultures containing a high proportion of EPCs (Fig. 3-G). Quantitative analysis was performed to determine potential tube length and to determine the number of branch nodes. The results suggested that ASCs did not promote or participate in tube formation by EPCs (Fig. 3H-I). In contrast to the classic HUVEC endothelial cell line, the EPCs exhibited limited capacity for *in vitro* tube formation.

### ***ASCs promote the angiogenesis of EPC through paracrine action***

As noted above, ASCs persistently expressed high levels of VEGFA and ANGPT1 during culture with EGM2 medium. ASCs may therefore promote the angiogenesis of EPCs through paracrine signaling. To test this, EPCs were seeded on matrigel in the presence of fresh control medium or supernatant from ASC or EPC cells cultured for 48 hours. Tube formation was analyzed after incubation for 4 hours. As shown in Fig. 4A, EPCs suspended in fresh EGM2 basal medium or EGM2 complete medium (containing growth factors optimized for endothelial cells) showed limited tube formation. This finding supports the results presented in Fig. 3. EPCs suspended in ASC or EPC culture medium generated typical vessel-like network. Quantitative analysis also confirmed significantly more proangiogenic activity in cultures with ASC and EPC medium, compared with controls (Fig. 4B). Further analysis indicated that, after culture for 48 hours, supernatant from ASC and EPC culture promoted the migration and invasion of EPCs seeded in transwell inserts (Fig. 5A). These results indicate that ASCs secrete a variety of growth factors that promote the angiogenesis of EPCs and/or the differentiation of EPCs into endothelial cells. EPCs may also have the ability to promote the angiogenesis via an autocrine mechanism.

To verify the ability of ASCs to regulate angiogenesis-related gene expression in EPCs, an *in vitro* coculture system with transwell chambers was established. The EPCs were cocultured with ASCs or empty control medium for 24 hours, then subjected to qPCR to measure the transcription of a panel of six potential angiogenesis markers. As shown in Fig. 6, coculture with ASCs significantly increased the transcription of angiogenic cytokines (VEGFA, angiopoietin-1, and angiopoietin-2), endothelial cell receptor tyrosine kinases (VEGFR1, TIE2) and endothelial cell adhesion molecules (CDH5) in EPCs, compared with controls. Notably, levels of VEGFR2, the main mediator of VEGF-induced endothelial proliferation and tubular morphogenesis, remained below the threshold required for detection. These findings suggest that binding to the Tie2 receptor by angiopoietins (ANGPT1 and ANGPT2) may play a more prominent role than VEGF in EPC angiogenesis. Moreover, the upregulation of endothelial cell adhesion molecules in EPCs suggests differentiation into a mature endothelial form.

### ***Co-transplantation of ASCs and EPCs promotes angiogenesis of adipose tissue grafts in vivo***

ASC and EPC are reported to promote the survival of adipose tissue grafts by increasing vascularization. In considering the proangiogenic effects of ASCs on EPCs *in vitro*, we speculated that the combination of ASCs and EPCs might be more effective in promoting the vascularization of adipose grafts than use of either cell type alone. To test this, intact adipose blocks were transplanted subcutaneously into nude mice. Tissue blocks were embedded in matrigel seeded with EPCs, a combination of ASCs and EPCs (1:1 ratio), or no cells. As shown in Fig. 7B-D, the appearance of adipose grafts dissected at 1 month post-implantation differed among groups. Only the grafts seeded with a combination of EPCs and ASCs were found to have adequate blood supply, with thick blood vessels extended into the grafts. In contrast, grafts containing no cells or EPCs alone were consistently more whitish in color, with thin vascular structures only on the graft surface. To evaluate the vascular density in explanted adipose grafts, histological sections of each group were stained with hematoxylin/eosin (H&E), which binds to vessels with large lumens containing blood elements. As shown in Fig. 7B-D, typical vascular structures with multilayered vascular walls and erythrocytes were commonly seen in grafts containing EPCs or the combination of ASCs and EPCs, but not in grafts that were not seeded with ASCs or EPCs. Quantitative analysis revealed that the vascular density in grafts containing ASCs and EPCs was significantly higher than that of grafts containing only EPCs (Fig. 7E). Generally speaking, these results indicate that ASCs may cooperate with EPCs to promote vascularization in transplanted adipose tissue.

## Discussion

The low survival rate and absorption of transplanted fat have posed problems for clinicians. The uncertainty of the rate of absorption in patients who have undergone fat transplantation means that plastic surgeons may administer too many injections or inject excessive amounts of fat [4,5,6]. Low rates of fat survival and absorption are generally associated with poor vascularization after transplantation.[7]

Studies have shown that ASCs promote angiogenesis in the context of ischemia. ASCs can differentiate into other types of cells, such as endothelial cells, vascular smooth muscle cells, and cardiomyocytes [8,9,10]. They also secrete a variety of functional growth factors and cytokines that promote angiogenesis (angiogenin, VEGF, HGF, bFGF, B-NGF) [11,12]. The results of *in vitro* experiments have shown that these cytokines can promote the survival of endothelial cells under hypoxic conditions [13]. Over the past decade, efforts to develop adipose tissue-mediated regenerative therapies have focused on ASCs. Mechanistic studies of ASC-based therapy have shown that paracrine secretion is responsible for the efficacy of these therapies. The beneficial paracrine effects of ASCs have been demonstrated in multiple clinical trials and basic studies of angiogenesis, immunomodulation, and tissue regeneration [14,15]. Mechanistic studies have demonstrated that the therapeutic proangiogenic effects of ASCs are attributed more to their secretory potential than to their capacity for multidifferentiation.

EPCs not only form blood vessels *in vitro* but also function as the building blocks of blood vessels *in vivo*. Many studies [16-18] have shown that transplanted EPCs can migrate to the ischemic or injured site. EPCs that have migrated secrete cytokines or differentiate into endothelial cells to participate in the construction of new blood vessels. The new blood vessels formed can be integrated into the blood

vessels of the host and then participate in nutrient transport. When vascular endothelial damage occurs in the body, EPCs located in the bone marrow can be mobilized to the injured site. The mobilized EPCs secrete a variety of angiogenic factors and differentiate into vascular endothelial cells, which promote vascular repair and neovascularization [19]. An *in vitro* three-dimensional (3D) network model was established to study the role of EPCs as a source of angiogenic factors. The results confirmed that EPCs can secrete VEGF and participate in vascular repair and neovascularization.

In 2007, Yoshimura *et al.* [20] reported that the use of stromal vascular fraction (SVF)-rich fatty stem cell-assisted fat transplantation for breast augmentation surgery achieved good results. In 2009 [21], autologous cell-assisted lipotransfer (CAL) technology was proposed. CAL technology mixes autologous ASCs with the adipocytes to be used for transplant. Since the introduction of CAL technology, scholars have conducted numerous basic and clinical research studies on the mechanisms underlying the survival of transplanted fat. ASCs appear to play a major role. ASCs promote vascular regeneration in transplanted fat, including VEGF. ASCs promote fat regeneration and blood vessel formation by differentiating into adipocytes and vascular endothelial cells. However, further research is needed. Paracrine signaling among ASCs may also aid in the treatment of ischemic disease [22,23,24,25,26]. Although there is strong evidence demonstrating the therapeutic effect of ASCs, the mechanisms underlying these effects are still unclear. The studies published to date have revealed two major mechanisms: multidifferentiation and paracrine secretion. A significant proportion of the benefits of ASCs may be due to secretion rather than differentiation[27].

The purpose of this study was to explore the relationship between ASCs and EPCs during angiogenesis. We cocultured ASCs and EPCs at different ratios. We found that the relative abundance of EPCs and ASCs in a given coculture resulted in significant differences in angiogenic capacity. We used the formation of tubular networks as an index for angiogenic capacity. As the proportion of ASCs increased, the ability of EPCs to form blood vessels decreased.(Fig. 3). These results indicate that ASCs do not directly participate in EPC tube formation *in vitro*. To investigate whether ASCs could promote the angiogenesis of EPC through a paracrine effect, we extracted the supernatant of ASCs cultured for 48 h. The results showed that ASC supernatant was more effective than control or EPC supernatant in promoting blood vessel formation by EPCs (Fig. 4). These results indicate that ASCs promote EPC tube formation by secreting growth factors. We next sought to investigate how ASC supernatant promoted angiogenesis among EPCs. To that end, we conducted EPC invasion and migration experiments. The results of these experiments showed that the supernatant from EPC culture as well as the supernatant from ASC culture promoted the migration and invasion of EPCs. However, the effect of the ASC supernatant on EPC migration and invasion was greater than that of the EPC supernatant (Fig. 5). These findings confirmed that ASCs exert a chemotactic effect on EPCs by secreting growth factors. Under hypoxic conditions, ASCs may recruit more EPCs through chemotaxis, promoting the formation of blood vessels and decreasing tissue hypoxia. We measured gene expression in EPCs incubated with ASC-conditioned medium. The results showed increased levels of ANGPT1, ANGPT2, CDH5, FLt1, Tie2, and VEGF (Fig. 6). The expression of these factors may allow EPCs to repair vascular damage by forming new blood vessels to decrease tissue ischemia. These findings support the hypothesis that ASCs recruit

EPCs through chemotaxis. ASCs appear to communicate in a paracrine fashion through the secretion of cytokines that promote the repair of blood vessels and the generation of new blood vessels through the upregulation of angiogenic factors.

We evaluate the effects of ASCs and EPCs on the survival of transplanted fat cells in nude mice. We performed tissue staining to visualize angiogenesis of the transplanted tissue. One month after implantation, the results of immunohistochemical analysis showed that blood vessels had grown into adipose tissue in the experimental group but not in the control group. (Fig. 7). *In vivo* angiogenesis is a complex multi-step event that requires the development of endothelial buds, their transformation to a capillary plexus, followed by maturation of the vascular network [28,29,30]. These processes depend on the interaction between EPCs and pre-existing blood vessels. The early angiogenesis observed in adipose tissue grafts seeded with ASCs and EPCs is thought to be mediated by various angiogenic growth factors, which initiate new blood vessel formation and vascular remodeling. ASCs may communicate in a paracrine fashion by secreting multiple growth factors to promote EPC-mediated blood vessel formation.

This study had some limitations. The molecular mechanism of the beneficial role of ASCs-EPCs in fat transplantation requires further elucidation. The results of this animal model should be verified in clinical trials. These limitations should be addressed in subsequent studies.

In summary, our results show that the transplantation of fat in combination with ASCs and EPCs can promote the survival of grafts. ASCs can regulate the expression of angiogenic factors in EPCs. These angiogenic factors act as paracrine signals to promote neonatal angiogenesis, suggesting that the use of ASCs and EPCs in combination with fat transplantation can promote the survival of grafts and the mechanism of neovascularization.

## Conclusions

This study found that the early EPC has limited ability of tube genesis and secretion of angiogenic factors. In contrast, Autogenous ASC expressed significantly higher levels VEGF $\alpha$  and angiopoietin1 under normal cultivation. *In vitro* studies showed that ASC conditioned medium could promote the invasiveness and migration capability of EPC, as well as tube genesis ability of EPCs. The coculture model further demonstrated ASC promote the expression of a series of angiogenesis marker genes in EPC, which indicated that the ASC may have the ability of promote the maturation or angiogenic potency of EPC through secretion of VEGF $\alpha$  and angiopoietin1. The *in vivo* transplant of fat tissues also demonstrated that coimplantation with ASC and EPC promote significantly higher levels of vascularization of adipose grafts than the implantation with EPC. In this study, we not only demonstrated the superiority of coimplantation of ASC and EPC in fat transplantation but also suggested a potential mechanism.

## Abbreviations

|            |  |
|------------|--|
| ASC        | adipose-derived stromal/stem cell                  |
| EPC        | endothelial progenitor cell                        |
| VEGF       | vascular endothelial growth factor                 |
| HGF        | hepatocyte growth factor                           |
| PB-EPC     | Peripheral blood adipose-derived stromal/stem cell |
| PBMC       | Peripheral blood mononuclear cell                  |
| DiI-Ac-LDL | DiI-acetylated low-density lipoprotein             |
| UEA-1      | Ulex europaeus agglutinin-1                        |
| VEGFR2     | vascular endothelial growth factor receptor 2      |
| PBS        | phosphate-buffered saline                          |
| BSA        | bovine serum albumin                               |
| RBC        | red blood cell                                     |
| qRT-PCR    | Quantitative real-time PCR                         |
| ANGPT1     | Angiopoietin-1                                     |
| EPC-MSC    | endothelial progenitor cells-mesenchymal stem cell |
| GAPDH      | glyceraldehyde-3-phosphate dehydrogenase           |
| VSF        | vascular fraction                                  |
| H&E        | hematoxylin/eosin                                  |

## Declarations

### Ethics approval and consent to participate

Animal use and care were in accordance with the animal care guidelines, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, publication number 8523, revised 1996) and the Care and Use Guidelines of Experimental Animals established by the Ministry of Medicine of Yunnan, China.

### Consent for publication

Not applicable

### Availability of data and material

All the data were generated during the study

## Competing interests

No competing interests

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## Authors' contributions

Xian Zhao designed the research; Fengshan Gan, Qing-Zhu Zhou, Wenli Huang, Xinwei Huang, performed the research; Liu Liu, Xinwei Huang, Xian Zhao analyzed the data; Fengshan Gan and Liu Liu wrote the paper. Fengshan Gan and Liu Liu contributed equally to this paper.

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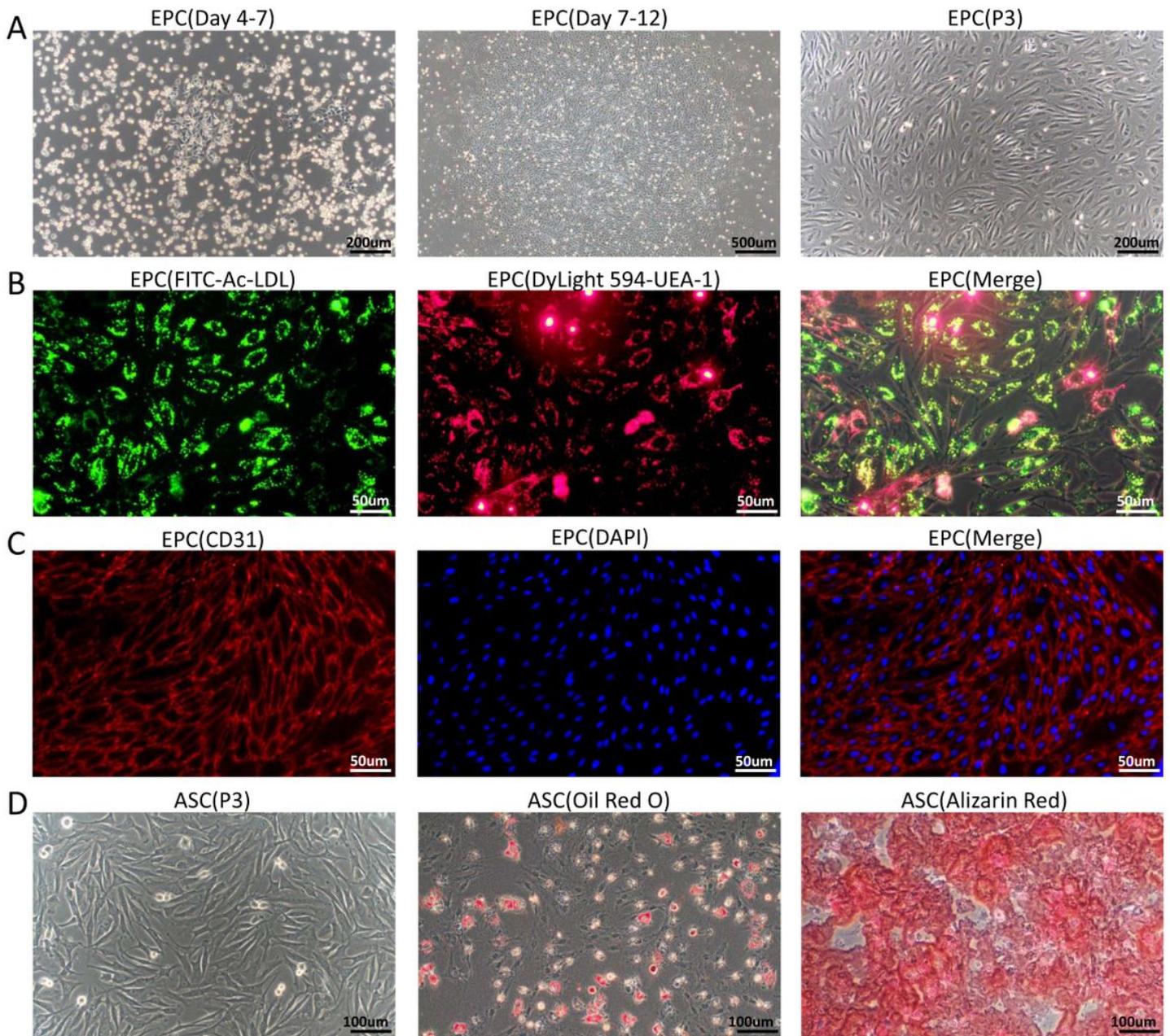
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## Table

Table 1. Primer pairs for quantitative RT-PCR analysis of rabbit target gene.

| Target gene                 | Forward (F) and reverse (R) primer sequence | Product length | NCBI accession |
|-----------------------------|---|----------------|----------------|
| <b>CD31</b>                 | F: GCATTGGCAAGGTGGTGAAG                     | 130            | XM_008271715.2 |
|                             | R: TGGCAGCTCACTTCTATGGC                     |                | XM_008271716.2 |
| <b>CD34</b>                 | F: TCCCGGAAGACCTTGATTGC                     | 128            | XM_008268471.2 |
|                             | R: TAAGGGTCTTCGCCAGCC                       |                |                |
| <b>CD45</b>                 | F: AAGACAACGGTGCAGGAAGG                     | 134            | XM_008268693.2 |
|                             | R: CCCGGCAACAAACTTCTG                       |                |                |
| <b>vWF</b>                  | F: TGAAGCTCCAGCGCATTGAA                     | 146            | NM_001329088.1 |
|                             | R: ATCTGGCAAGGTCCACACAT                     |                |                |
| <b>VEGFA</b>                | F: AGTTCGAGGAAAGGGCAAGG                     | 157            | XM_017345155.1 |
|                             | R: ACGCGAGTCTGTGTTTTTGC                     |                |                |
| <b>VEGFR1</b>               | F: GTGCACGGTCAACAAGTTCC                     | 80             | XM_008275216.2 |
|                             | R: TGTGGCGCATTGTTCTGTTG                     |                |                |
| <b>VEGFR2</b>               | F: GGTGCTTCTCCGTATCCTGG                     | 222            | NM_001195670.1 |
|                             | R: GTAGTCTTTGCCACCCTGCT                     |                |                |
| <b>ANGPT1</b>               | F: TGTGCCCTCATGCTTACAGG                     | 236            | XM_008255827.2 |
|                             | R: CTCGGTCTCCAGATCGCTTC                     |                |                |
| <b>ANGPT2</b>               | F: TGGCTGGGAAACGAGTTTGT                     | 198            | XM_002720896.3 |
|                             | R: TGGTTGGCTGATGCTGCTTA                     |                |                |
| <b>Tie2</b>                 | F: CAAGAACAACCCGGATCCCA                     | 117            | XM_017340937.1 |
|                             | R: TCCTTCTTGATGCGTGCCTT                     |                |                |
| <b>CDH5</b>                 | F: CAGGTACGAGATCGTGGTGG                     | 120            | XM_008257382.2 |
|                             | R: GTCTGCGTGAAGATGGGGAA                     |                |                |
| <b>GAPDH<sup>[31]</sup></b> | F(PS182): TGACGACATCAAGAAGGTGGTG            | 120            | NM_001082253   |
|                             | R(PS183): GAAGGTGGAGGAGTGGGTGTC             |                |                |

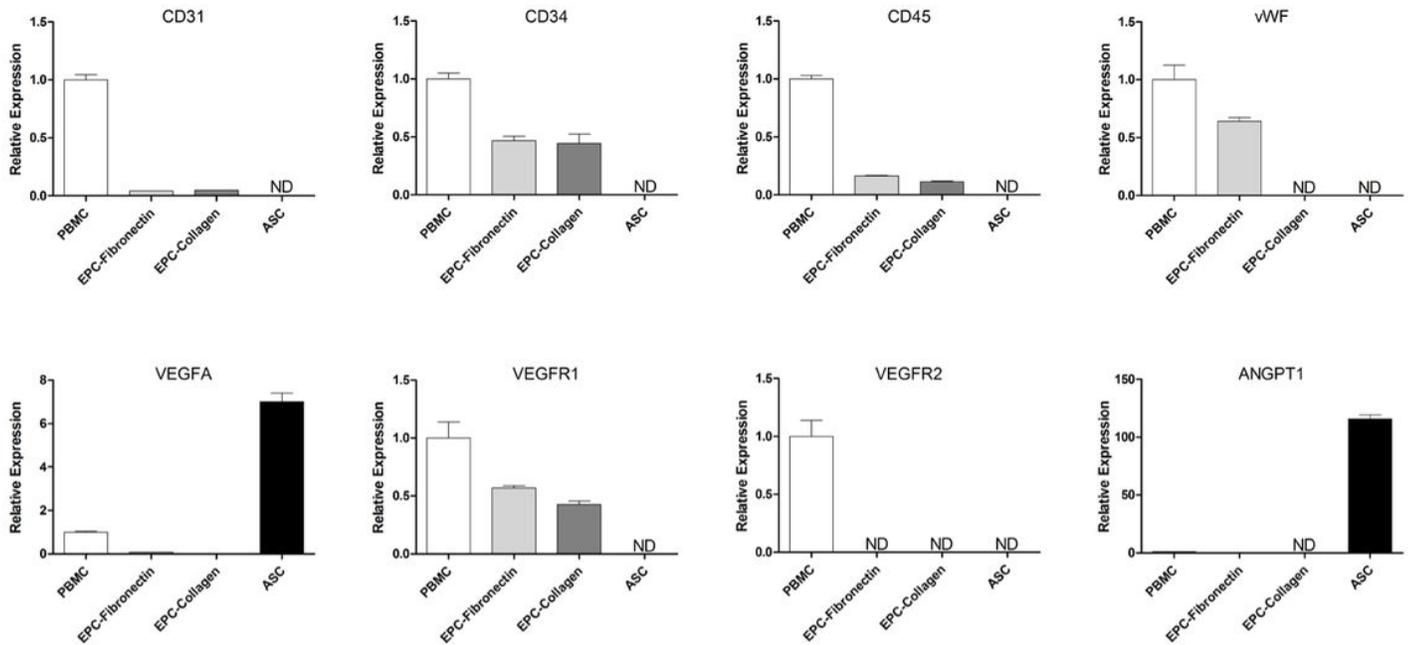
## Figures



**Figure 1**

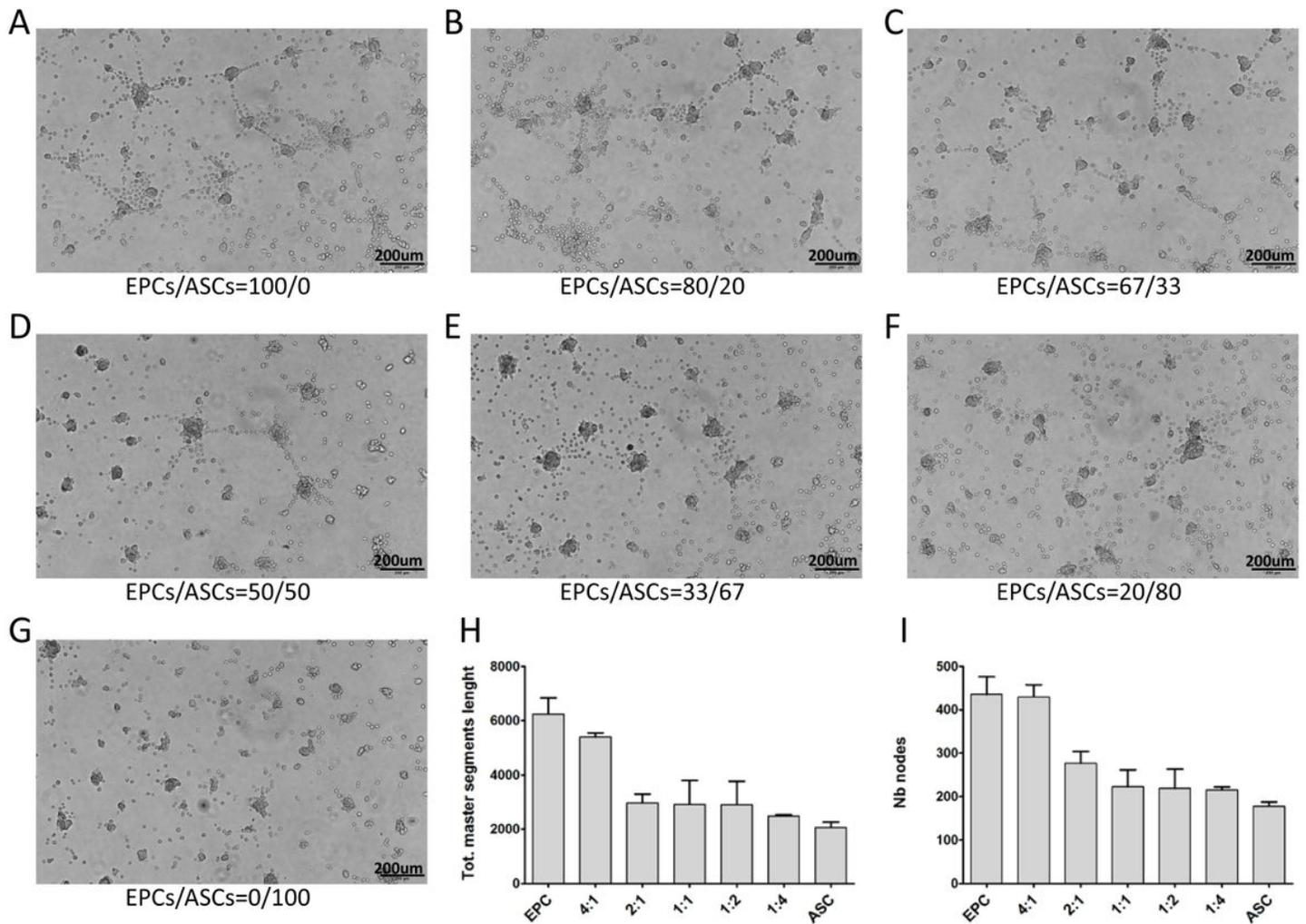
Isolation and characterization of rabbit EPCs and ASCs. (A) Rabbit peripheral blood mononuclear cells were seeded on plates pretreated with fibronectin and supplemented with EGM-2 complete medium. At 4 to 7 days, adherent cells with typical cobblestone-like morphology appeared. At 9-12 days, the EPCs expanded into colonies. Third-passage EPCs cultured in complete EGM-2 medium exhibited spindle-like morphology under inverted phase-contrast microscopy. (B) The functional characteristics of EPCs were assessed by testing their ability to take up FITC-Ac-LDL and bind to DyLight 594-UEA-1. The results were visualized with fluorescence microscopy. (C) Immunofluorescent staining revealed the expression of endothelial cell marker CD31 in EPCs. Cell nuclei are stained with DAPI. (D) Third-passage ASCs cultured in complete EGM-2 medium displayed fibroblast-like morphology. The multilineage differentiation potential of ASCs was assessed in vitro. Osteogenesis was assessed by staining with Alizarin Red to

detect the formation of calcium-rich deposits. Adipogenesis was assessed by staining with Oil red O to detect lipid vacuoles in the cytoplasm.



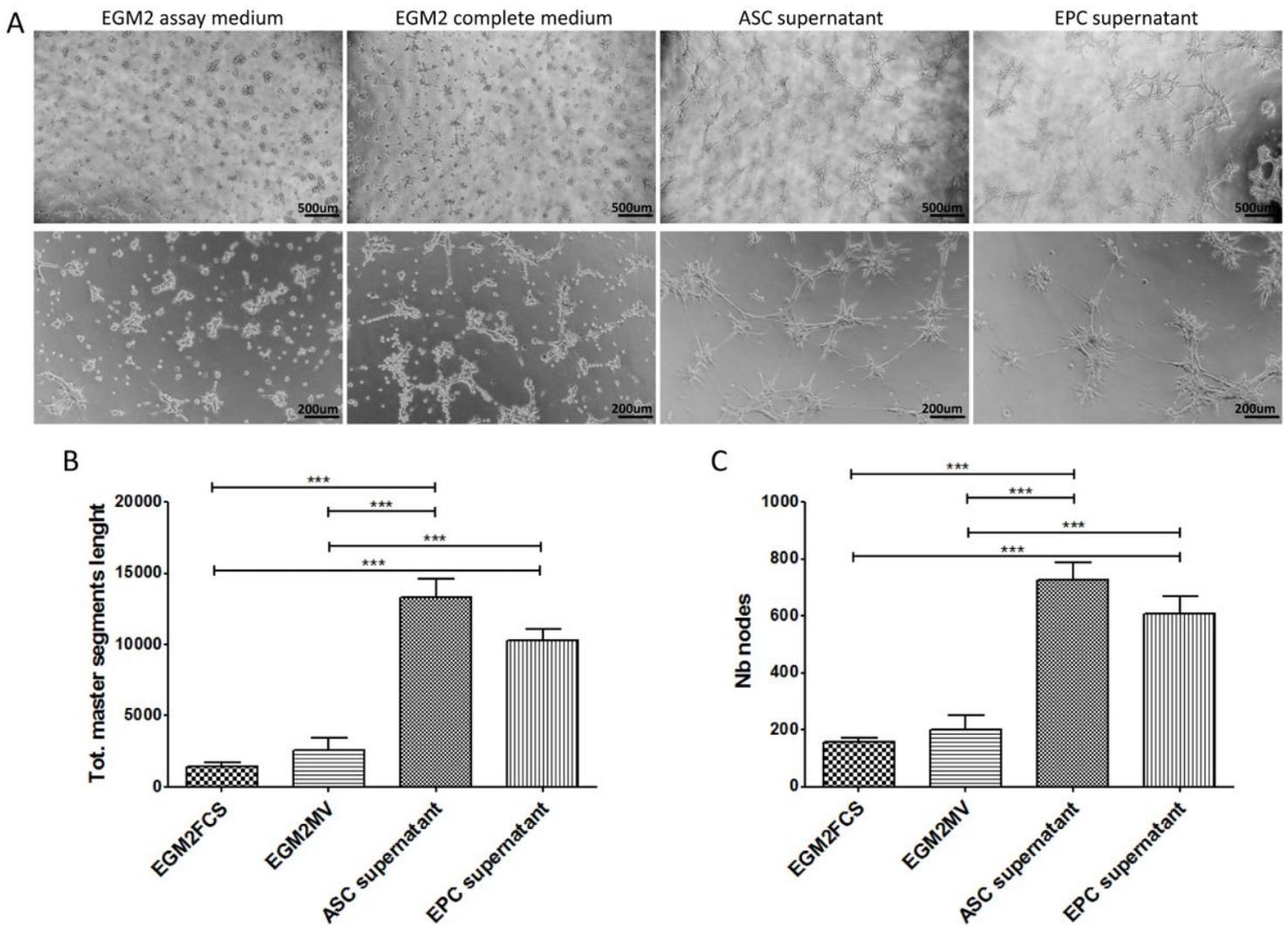
**Figure 2**

Molecular phenotyping of EPCs and ASCs isolated from rabbits. The relative expression of surface markers and angiogenic factors in PBMCs, EPCs, and ASCs was evaluated with qPCR. Levels below the detection limit are depicted as ND. Data are expressed as mean  $\pm$  standard errors of the mean (SEM).



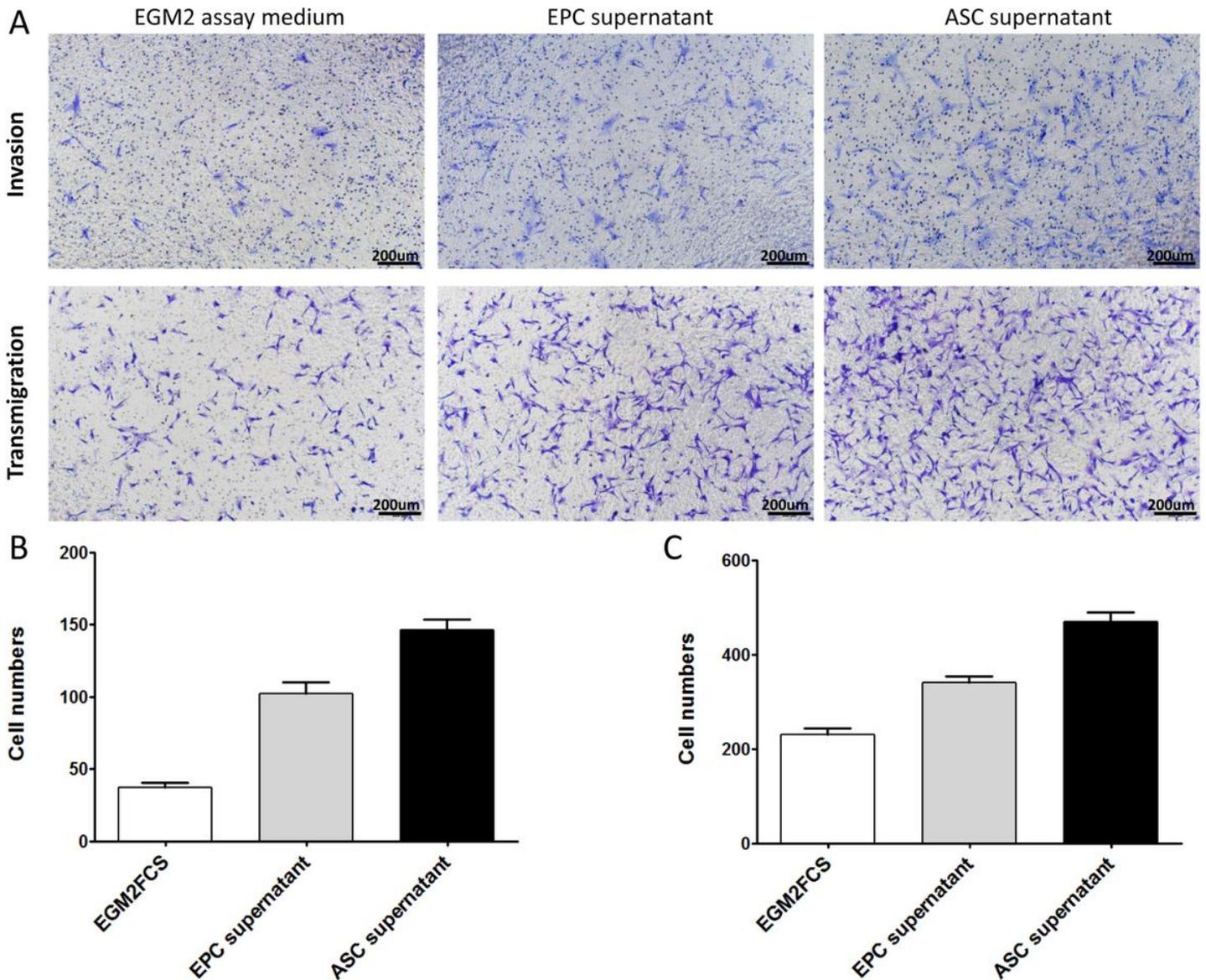
**Figure 3**

Tube formation in various ratios of cocultured EPCs and ASCs. (A-G) Cocultures prepared with various ratios of EPCs and ASCs were incubated for 4 h on matrigel-coated plates. Tube formation was analyzed using inverted phase-contrast microscopy. Each micrograph presents the results of 3 separate experiments. (H-I) Quantitative comparison of total master segment length (H) and number of formed branch nodes (I) in EPC-ASC cocultures. Data are presented as mean  $\pm$  SEM. \*,  $p < 0.05$ .



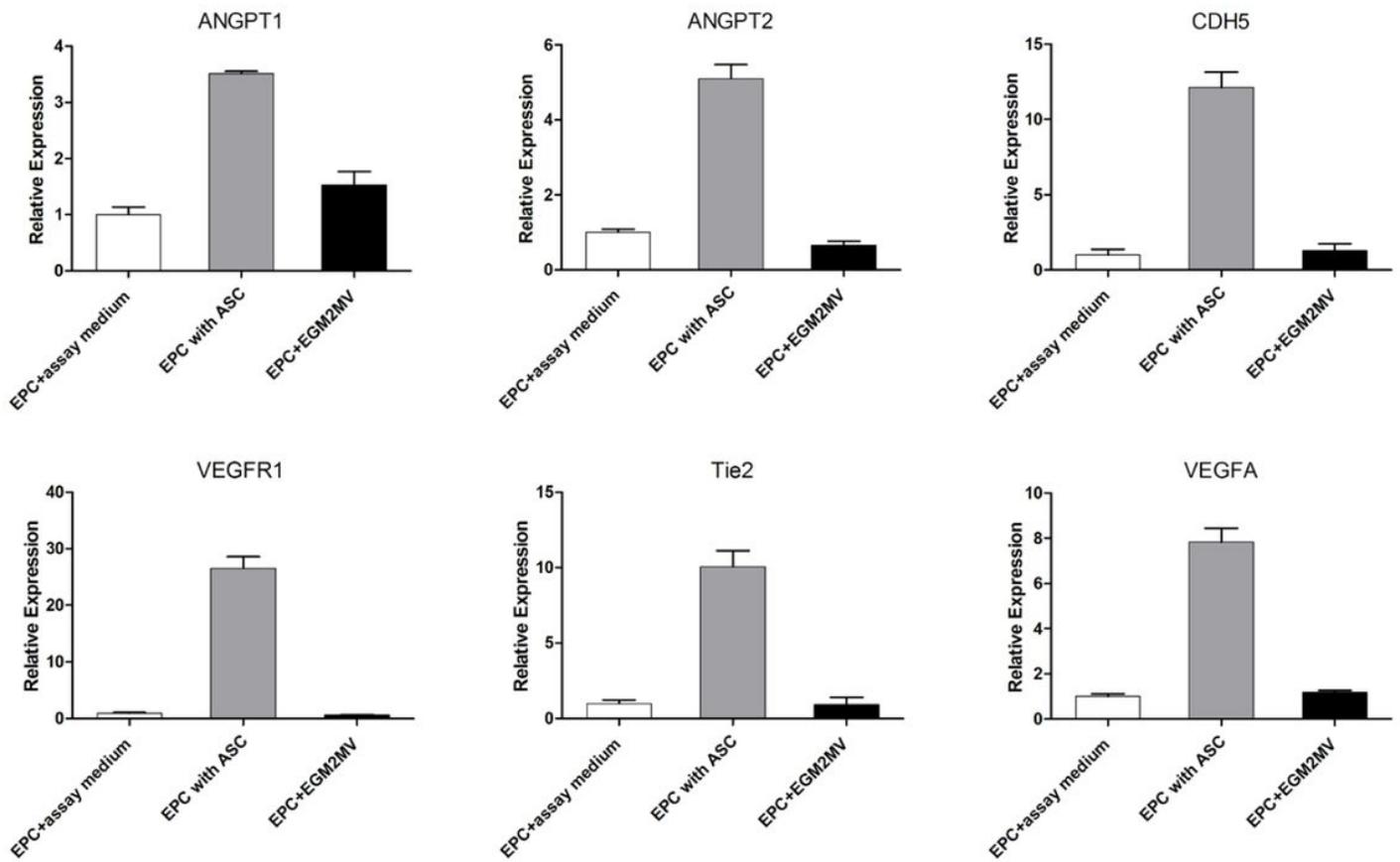
**Figure 4**

Supernatant from ASC cultures promotes EPC tube formation in vitro. (A) Representative images of EPC tube formation on matrigel in the presence of supernatant from ASCs or EPCs cultured for 48 hours. Fresh EGM2 assay medium was used as a negative control, and complete EGM2 medium was used as a positive control. Higher-magnifications views are presented (B-C). Total master segment length (B) and number of branch nodes (C) were calculated from 6 random fields in images of three wells in series. Data are presented as mean  $\pm$  SEM. \*\*\*,  $p < 0.001$ .



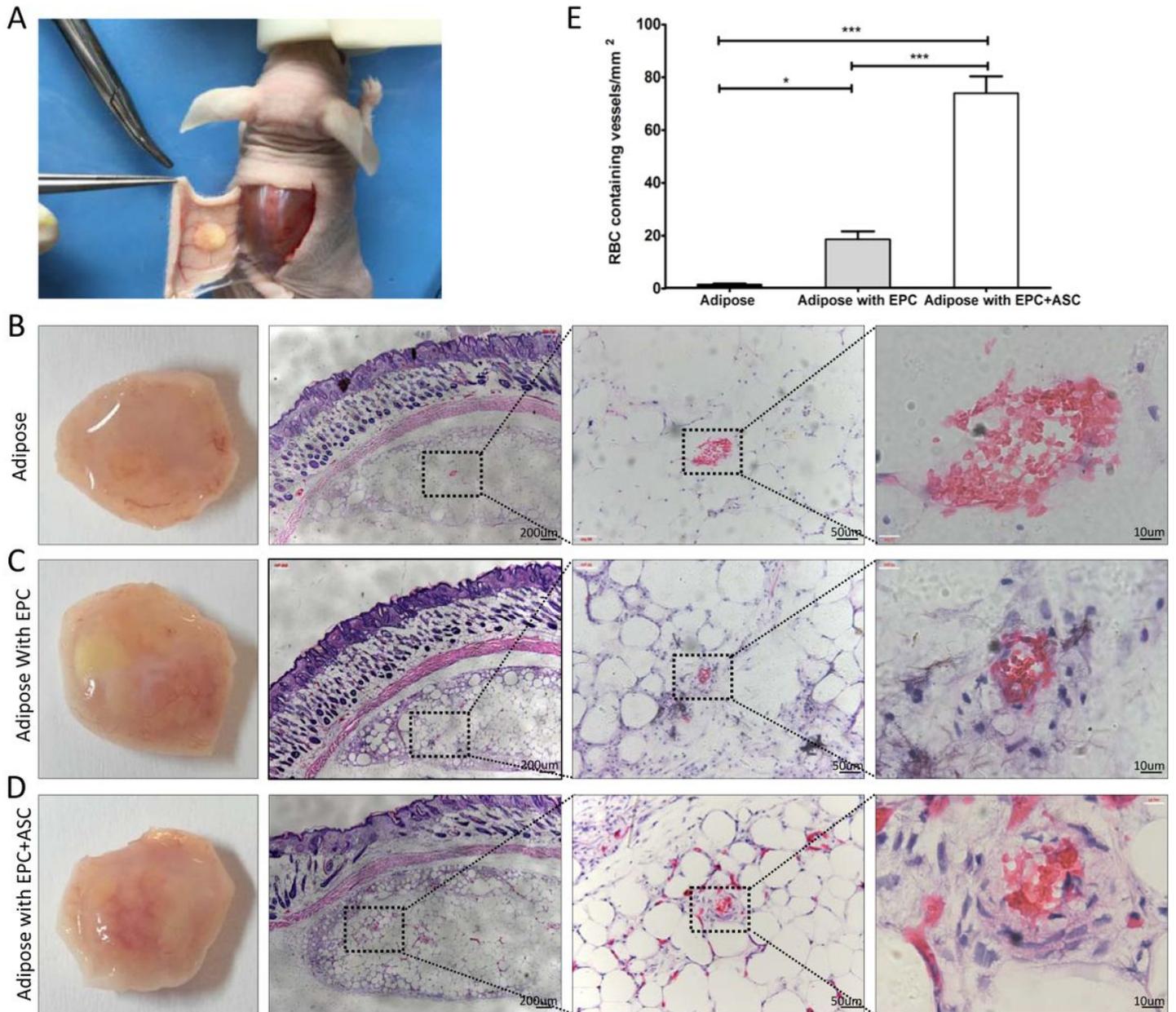
**Figure 5**

Supernatant from ASC cultures promotes EPC invasion and migration in vitro. (A) Representative images of EPC invasion and migration in the presence of EPC supernatant, ASC supernatant, or fresh EGM2 assay medium, respectively. Quantitative analysis of EPC invasion (B) and migration (C). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.01$ .



**Figure 6**

Coculture with ASCs promote the expression of angiogenesis markers in EPCs. EPCs were cocultured with ASCs or an equivalent volume of empty EGM2 assay medium or complete EGM2 medium as controls. After coculture for 24 hours, EPCs were harvested for qPCR detection of the expression of angiogenesis markers including VEGFA, ANGPT1, ANGPT2, CDH5, VEGFR1, and Tie2. Levels below the threshold for detection are depicted as ND. Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.01$ .



**Figure 7**

Coimplantation of ASCs with EPCs increases vascular density in adipose grafts. (A) Photograph illustrating the subcutaneous transplantation of adipose tissue in nude mice. In each group, the fat implants were harvested at 30 days postimplantation and stained with HE. (B-D) Representative photographs and images of HE-stained sections of implanted adipose tissue (B), implanted adipose tissue containing EPCs (C), and implants containing a combination of ASCs and EPCs (D). Higher-magnification images of the boxed areas are shown on the right. (E) The density of RBC-filled vessels in the implants of each group was analyzed. Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .