Gene profile of soluble growth factors involved in angiogenesis, in an adipose-derived stromal cell/endothelial cell co-culture, 3D gel model

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Abstract

Objectives: The aim of this study was to investigate gene expressions of growth factors for angiogenesis, in a three-dimensional (3D) gel populated with adipose-derived stromal cells (ASCs) and endothelial cells (ECs) in co-culture.

Materials and methods: The 3D gel, mixed with green fluorescent protein (GFP)-positive ASCs and DsRed-Express-positive ECs, 1:1 ratio, was established in vitro. The phenomenon of angiogenesis was observed using confocal microscopy. To detect gene expressions for growth factor proteins in both ASCs and ECs, transwell co-culture was used, and cell lysate samples were collected at 1, 3, 5 and 7 days. Semi-quantitative polymerase chain reaction (PCR) was conducted to quantify mRNA expressions of the growth factors.

Results: Angiogenesis was first observed in the gels by 7 days post-implantation. Over this time in ECs, genes coding for VEGFA/B, IGF-1, HIF-1 α , FGF-1/-2 and BMP-5/-7 significantly increased. Meanwhile in ASCs, genes coding for VEGFA/B, IGF-1, HIF-1 α , FGF-1/-2 and BMP-6 also were significantly enhanced. In particular, increased amounts of *IGF-1* and *HIF-1 α* in both ECs and ASCs were prominent relative to other factors.

Conclusions: Contact co-culture with ASCs and ECs at 1:1 ratio, in the 3D gel promoted angiogenesis; non-contact co-culture further confirmed gene expressions for growth factors, *VEGFA/B*, *IGF-1*, *HIF-1 α* and *FGF-1/-2* in both ASCs and ECs; *BMP-5/-7* in ECs and *BMP-6* in ASCs were also

confirmed. This establishment of growth factor expression seemed to be responsible for enhancement of angiogenesis. This indicates that these factors could be utilized as targets for engineered angiogenesis.

Introduction

Adipose-derived stromal cells (ASCs) can now be seen to be emerging as clinical options for treating tissue damage and diseases, because of their ability to differentiate into many different types of cell lineage and to promote neovascularization (1,2). Their successful demonstration for transplantation into animal models, highlights their importance and reliable effects (3). ASCs, widely obtained from adult adipose tissue, are able to replicate as well as undifferentiated cells, to develop into mature adipocytes and to differentiate into multiple other cell types, such as chondrocytes, myocytes and osteocytes (4). They also have been shown to differentiate into pancreatic beta-cells (5), myocardiocytes (6) and endothelial cells (ECs) (7), and thus generate considerable interest for their potential use in tissue engineering and regenerative medicine.

More recently, ASCs have been shown to secrete a wide range of trophic factors that can stimulate regeneration of tissues of multiple lineages (8). In angiogenesis, ASCs are thought to promote endothelial cell survival and proliferation, endothelial tubulogenesis, vascularization and stable vascular assembly (9). Combination of ASCs and ECs can further promote formation of vascular networks (10). Moreover, formation of functional microvascular beds, and co-implantation of endothelial cells and mesenchymal stromal cells (MSCs), which are similar to ASCs, provide possible methods for cell-based revascularization therapies, to treat various diseases, such as those of the peripheral vasculature and ischemic heart disease (11,12). However, as these potential cell therapies become more attractive to research

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and clinical procedures, their secretory profiles need to be more thoroughly investigated in terms of the roles stem cell-secreted factors have in tissue regeneration.

The bone morphogenetic protein (BMP) pathways play important roles in angiogenetic promotion and take part in hereditary vascular diseases (13,14). Some studies have indicated that BMP signalling has direct effects on angiogenesis with ECs, both *in vitro* and *in vivo*. BMP-6 enhances angiogenesis *via* cyclooxygenase 2-dependent prostanoid generation *in vitro* (15). BMP-2 induces angiogenesis during both bone formation and its repair (16). Noggin, an inhibitor of the BMP family, principally blocks the activation phase of angiogenesis (17). Also, dorsomorphin, inhibitor of both BMP and vascular endothelial growth factor (VEGF) signalling (18), has been reported to cause major defects in angiogenesis (19).

Thus, in this study, we first established a model of angiogenesis using a three-dimensional (3D) collagen gel, seeded with ASCs and ECs at 1:1 ratio. We then detected gene expressions of *VEGFA* and *VEGFB*, and investigated gene profiles that code for relevant growth factors in both ASCs and ECs. By confirming the key growth factors participating in promotion of angiogenesis, we have hoped to provide direct information concerning paracrine effects during formation of the vascular system.

Materials and methods

Cell culture

Animal materials used for this study were obtained according to governing ethical principles and our protocol was reviewed and approved by our Institutional Review Board (IRB).

ASCs were obtained from subcutaneous adipose tissue of 4-week female mice. Briefly, collected adipose tissue was cut into small pieces and treated with 0.75% type I collagenase, for 30 min. This ASC suspension was then collected and mixed 1:1 (v/v) with fresh 10% heat-activated foetal bovine serum (FBS) α -MEM (0.1 mM non-essential amino acids, 4 mM L-glutamine, 1% penicillin-streptomycin solution). The mixed suspension was centrifuged at 180 *g* for 5 min. After removing the first supernatant, 10% FBS α -MEM was added to centrifuge tubes to re-suspend the ASCs. Next, the ASCs in suspension were seeded into plates or flasks at 37 °C in a humidified atmosphere of 5% CO₂ until use. Purified ASCs could then be obtained after two passages as described previously (20).

To obtain purified ECs at high density, brain microvascular tissue was collected from neonatal mice.

Samples were cut into small pieces and treated with 0.5% type II collagenase for 1 h. The ECs suspension was collected and mixed 1:1 (v/v) with fresh 10% heat-activated FBS DMEM (high-glucose DMEM, 0.1 mM non-essential amino acids, 4 mM L-glutamine, 1% penicillin-streptomycin solution) and the mixed suspension was centrifuged at 179 *g* for 8 min. After removing the supernatant, remaining tissue fragments were mixed with 20% bovine serum albumin (BSA) and centrifuged at 1000 *g* for 20 min. Once more the supernatant was removed and fresh 10% FBS DMEM was added to centrifuge tubes to re-suspend the ECs again. ECs were then seeded into plates or flasks at 37 °C in a humidified atmosphere of 5% CO₂ until use.

To obtain green fluorescent protein-positive ASCs and DsRed-Express-positive ECs, subcutaneous adipose tissue and brain microvascular tissue were collected from enhanced green fluorescent protein (GFP) transgenic mice (Centre for Genetically Engineered Mice, West China Hospital, Sichuan University, Chengdu, China) and DsRed-Express transgenic mice (Genetic Centre of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Centre of Comparative Medicine, Peking Union Medical College, Beijing, China), respectively. Cell isolation for these was as described above.

Co-culture

Cell-cell co-culture of GFP-ASCs and ECs labelled with red fluorescent protein (RFP) was used to be able to view morphology of vessel-like structures in our 3D collagen gel model. ASCs and ECs were mixed in a 1:1 ratio and suspended in DMEM-HG (Hyclone, Logan, UT, USA) and rat tail tendon type I collagen (Shengyou Biotechnology, Hangzhou, China). Seeded cells were transferred to 96-well plates to form 3D gel samples at 37 °C, and cultured for 1–2 weeks.

To detect gene profiles of the growth factors in the ASCs and ECs, here we used transwell co-culture. Briefly, ECs were seeded on six-well plates, $1-5 \times 10^6$ cells per well (85–95% confluence) and were allowed to equilibrate for 24 h. Culture media were then replaced with 2% FBS DMEM for 12 h starvation. Then the ECs were split into two groups. For EC mono-culture, media were replaced with fresh 1% FBS DMEM; meanwhile, the transwell chamber with no ASCs was filled with 1% FBS α -MEM (culture medium control). In the EC co-culture, media were replaced with fresh 1% FBS DMEM and the transwell chamber was also filled with 1% FBS α -MEM; however here, ASCs were synchronously seeded into the chamber at the beginning

of EC seeding. EC cell lysate samples (1000 µl) were collected at 1, 3, 5 and 7 days after co-culture. ASCs were seeded on six-well plates and ECs were seeded into transwell chambers.

Confocal laser scanning microscopy (CLSM) of the 3D model co-culture

For morphologies of normal and fluorescent ASCs and ECs, images were captured using an IX71 inverted microscope (Olympus, Tokyo, Japan).

For the morphology of vascular-like structures, 3D collagen gel images were scanned using a Leica DMIRE2 confocal laser scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany, parameter: 20×, Leica Microsystems original image: 1024 × 1024, 100 µm) equipped with a x60 oil immersion objective lens. Image analysis software Imaris 7.0.0 (Bitplane, Zurich, Switzerland) was used for three-dimensional reconstruction.

Semi-quantitative polymerase chain reaction (PCR)

RNA samples from ASCs and ECs were isolated using the RNeasy Plus Mini Kit (Qiagen, Shanghai, China) with genomic DNA eliminator. Isolated RNA was dissolved in RNase-free water and quantified by measuring absorbance at 260 nm using a spectrophotometer. RNA samples were then treated with DNase I (Mbi, Glen Burnie, MD, USA), and cDNA was prepared from each sample, using 0.5 µg total RNA and cDNA synthesis kit (Mbi) at 20 µl final volume.

To evaluate expression levels of growth factors in different treated groups as normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (β -actin), semi-quantitative PCR was performed using a PCR kit (Mbi) and thermo-cycler (Bio-Rad, Hercules, CA, USA). Selected sets of primers are shown in Table 1. BLAST was used to search for all primer sequences to ensure gene specificity. Semi-quantitative PCR were performed in 25 µl volume containing 1 µl cDNA sample. PCR program consisted of 30 s denaturation cycle at 94 °C, 30 s annealing cycle at 55–65 °C and 72 °C, 30 s elongation cycle, 22–28 amplification cycles. Products were resolved by 2% agarose gel electrophoresis in trisborate/ethylenediamine-tetraacetic acid (EDTA) buffer, and visualized by staining with ethidium bromide.

Quantification of gene changes to angiogenesis-related growth factors in ECs and ASCs after 7 days co-culture, was calculated using the optical density (OD) method, with Quantity One 4.6.3 software (Bio-Rad) based on the semi-quantitative PCR.

Table 1. Primers of housekeeping genes (*GAPDH* & β -actin) and related growth factor genes designed for semi-quantitative PCR

mRNA	Primer pairs
<i>GAPDH</i> (233 bp)	Forward GGTGAAGGTCGGTGTGAACG Reverse CTCGCTCCTGGAAGATGGTG
β -ACTIN (266 bp)	Forward GTCCCTCACCTCCCAAAAG Reverse GCTGCCTCAACACCTCAACCC
<i>VEGFA</i> (106 bp)	Forward CTGCTGTGGACTTGTGTTGG Reverse AAAGGACTTCGGCCTCTCTC
<i>VEGFB</i> (128 bp)	Forward GCAACACCAAGTCCGAATG Reverse CTGGCTTACAGCACTCTCC
<i>TGF-β_1</i> (113 bp)	Forward TGGAGCCTGGACACACAGTA Reverse TAGTAGACGATGGGCAGTGG
<i>IGF-1</i> (171 bp)	Forward CTGCTTGCTCACCTTCACC Reverse TCATCCACAATGCCTGTCTG
<i>HIF-1α</i> (129 bp)	Forward TGAACATCAAGTCAGCAACG Reverse CACAAATCAGACCAAGCAC
<i>EGF</i> (112 bp)	Forward GCTCTTCTGGGTTCAGGACA Reverse AGACAAACTGTGCCGTGCTT
<i>VE-ca</i> (102 bp)	Forward CATCGCAGAGTCCCTCAGTT Reverse TCAGCCAGCATCTTGAACCT
<i>PGF</i> (107 bp)	Forward CCGATAAAGACGCCAACATC Reverse CATTACAGAGCACATCCTGA
<i>FGF-1</i> (124 bp)	Forward CCACAGCCCAGCAGTTATC Reverse CTCCTACGCCCACTCTTCAG
<i>FGF-2</i> (138 bp)	Forward AGGAAGATGGACGGCTGCT Reverse GCCCAGTTCGTTTCAGTGC
<i>BMP-2</i> (106 bp)	Forward TGAGGATTAGCAGGTCTTGC Reverse CGTTTGTGGAGCGGATGT
<i>BMP-4</i> (103 bp)	Forward TCTTCAACCTCAGCAGCATC Reverse AAGCCCTGTTCCAGTCAG
<i>BMP-5</i> (106 bp)	Forward CACCAGGGAAACAAGCATCT Reverse CTCACCAAATACTCCGACTCCT
<i>BMP-6</i> (106 bp)	Forward AGGTCCATTCCAGCAAG Reverse TCACACCACCGAGTCAAC
<i>BMP-7</i> (106 bp)	Forward TCCTGCATCCACAAAGAA Reverse TTCCAGGGACACAGCATGA

Statistical analysis

All experiments were performed in triplicate and reproduced at least three separate times. Statistical analysis of data was performed with SPSS 16.0 (IBM, Silicon Valley, CA, USA) using one-way ANOVA, to compare means of all groups, and Student–Newman–Keuls (SNK-q) testing to compare means of each two groups. Data were considered significantly different with two-tailed *P* value <0.05.

Results

Co-culture promoted formation of vessel-like structures in 3D gels

First, mECs and mASCs were successfully isolated from normal and fluorescent protein-labelled mice (Fig. 1a). ECs were identified using factor VIII (FVIII)

immunofluorescence (unpublished data) and ASCs were identified to be positive for stromal cell markers CD34, CD146, Sca-1 and CD44, with capabilities of adipogenic and osteogenic differentiation (13). In the *in vitro* 3D gels, RFP-mECs alone formed vessel-like structures after 7 days culture (Fig. 1b mono-culture group), but co-culture of RFP-mECs and GFP-mASCs significantly enhanced formation of vessel-like structures (Fig. 1b co-culture group). Furthermore, lengths of vessel-like structures were far greater in the co-culture group compared to the mono-culture group (up to 1.954-fold), and connection formation of vessel-like structures was higher in co-cultured groups compared to mono-culture groups (up to 3.141-fold) (Fig. 1c).

VEGF genes were up-regulated in both ECs and ASCs after co-culture

To explore expression of variation of angiogenesis-related genes in co-cultured ECs and ASCs, transwell chambers were used to achieve gene detection in both cell types

after crosstalk. By 1, 3, 5 and 7 days post semi-quantitative PCR, we found that *VEGFA* and *B* in ECs were up-regulated after transwell co-culture with ASCs (Fig. 2-EC group). After quantification using the OD method with Quantity One 4.6.3 software (Bio-Rad), we found *VEGFA* and *B* were 1.154-fold and 2.088-fold higher in co-cultured groups compared to mono-culture groups (Table 2-total quantification), respectively. Of the ASCs, basal expression of *VEGFA* was higher than that in EC groups but *VEGFB* was lower. After transwell co-culture, we also found that both *VEGFA* and *B* in ASCs were enhanced compared to mono-culture ASCs (Fig. 2-ASC group) and quantities were up to 1.339 and 1.429-fold higher, respectively.

Relevant growth factors were modulated in both ECs and ASCs after co-culture

We then determined gene expressions for those of growth factors related to angiogenesis, in both ECs and ASCs, after transwell co-culture (Fig. 3). Compared to

Figure 1. Co-culture promoted angiogenesis in 3D collagen gels. (a) Morphology of normal cultured, and fluorescent adipose stromal cells (ASCs) and endothelial cells (ECs) *in vitro*. GFP-mASC = ASCs from the green fluorescent protein-labelled mouse; RFP-mEC = ECs from the red fluorescent protein-labelled mouse. ASCs shown were at passage III, and ECs were primary cells. (b) Angiogenesis in 3D gel after co-culture between ASCs and ECs, was significantly enhanced after 7 days. Mono-culture ECs in 3D gel after 7 days culture; co-culture group with mixed cell culture in 3D gel, after 7 days culture. (c) Analyses of vessel-like structure lengths, and vessel-like connection formations, using Image-Pro Plus Software 6.0 (Media Cybernetics, Rockville, MD, USA).

Figure 2. *VEGFA/B* genes in ECs and ASCs after co-culture, by semi-quantitative PCR. *GAPDH* and β -actin were the reference genes; samples taken after 1, 3, 5 and 7 days. Gels shown are representative of three independent experiments ($n = 3$).

Table 2. Gene changes of angiogenesis-related growth factors in ECs and ASCs after co-culture. The fold changes were calculated by OD method with Quantity One 4.6.3 software (Bio-Rad) based on the semi-quantitative PCR at 7 day

Gene profile of growth factors	Endothelial cells		Adipose Stromal cells	
	Fold changes		Fold changes	
<i>VEGFA</i>	1.154	↑*	1.339	↑*
<i>VEGFB</i>	2.088	↑*	1.429	↑*
<i>TGF-β_1</i>	1.056		0.903	
<i>IGF-1</i>	1.287	↑*	7.285	↑*
<i>HIF-1α</i>	8.519	↑*	2.129	↑*
<i>EGF</i>	—		—	
<i>VE-CA</i>	0.870		1.443	↑*
<i>PDGF</i>	1.115		1.088	
<i>FGF-1</i>	1.236	↑*	1.651	↑*
<i>FGF-2</i>	1.653	↑*	2.062	↑*
<i>BMP-2</i>	1.152		—	
<i>BMP-4</i>	0.832		—	
<i>BMP-5</i>	1.585	↑*	—	
<i>BMP-6</i>	1.144		2.355	↑*
<i>BMP-7</i>	1.933	↑*	—	

*Significant difference with respect to the mono-culture group ($P < 0.05$).

mono-culture ECs, we found that in co-cultured EC groups, transforming growth factor-beta₁ (*TGF- β_1*) was slightly higher; platelet-derived growth factor (*PDGF*) and VE-caderin showed no significant variation between mono- and co-cultures; epidermal growth factor (*EGF*) was not expressed in ECs; insulin-like growth factor-1 (*IGF-1*), hypoxia inducible factor-1 α (*HIF-1 α*), fibroblast growth factor-1 (*FGF-1*) and *FGF-2* all had significant increments (*IGF-1* was as high as up 1.287-fold in co-culture groups relative to mono-cultures, *HIF-1 α* was up 8.519-fold, *FGF-1* up 1.236-fold, and *FGF-2* up 1.653-fold; Table 2 for total quantification). Compared to mono-cultured ASCs (Fig. 3, right lane), we observed that in co-cultured ASCs, *TGF- β_1* and VE-caderin had no significant variation and *EGF* is not detectable. Other

growth factors, *IGF-1*, *HIF-1 α* , *PDGF*, *FGF-1* and *FGF-2*, all increased to different levels (*IGF-1* as high as 7.285-fold in co-culture groups relative to mono-culture groups, *HIF-1 α* up 2.129-fold, *FGF-1* up 1.651-fold and *FGF-2* up 2.062-fold; Table 2-total quantification). From these results, we also found that basal expressions of *IGF-1*, VE-caderin, *PDGF* and *FGF-1* were higher in ECs than in ASCs but *HIF-1 α* and *FGF-2* were lower in ECs than in ASCs.

BMP family modulation in both ECs and ASCs after co-culture

We next examined gene variations coding for BMP family members (Fig. 4). In general, *BMPs* were expressed in ECs but little in ASCs except for *BMP-6*. In EC groups, *BMP-2*, -4 and -6 showed no significant changes, but *BMP-5* and -7 were higher in co-cultured groups than in mono-cultures (up to 1.585-fold and 1.933-fold, respectively; Table 2 – total quantification). In ASC groups, the only expressed member, *BMP-6*, was significantly enhanced in co-cultured groups than in mono-cultures (up to 2.355-fold; Table 2 – total quantification).

Discussion

Stem cell candidates used in tissue engineering and regenerative medicine include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and post-natal adult stem cells. ESCs are capable of extensive self-renewal and have potential to differentiate into

Figure 3. Gene profiles coding for growth factors, *TGF- β_1* , *IGF-1*, *HIF-1 α* , *EGF*, *Ve-cadherin*, *PGF*, *FGF-1* and *FGF-2*, in ECs and ASCs, after co-culture, by semi-quantitative PCR. Samples taken after 1, 3, 5 and 7 days. Gels shown are representative of three independent experiments ($n = 3$).

Figure 4. Gene expressions of BMPs in ECs and ASCs, after co-culture, by semi-quantitative PCR. Samples taken after 1, 3, 5 and 7 days. Gels shown are representative of three independent experiments ($n = 3$).

any type of somatic tissue type (21,22). iPSCs can be derived from differentiated cells such as fibroblasts and appear to have the same potential and properties as ESCs, but their generation is not dependent on a source of embryos (23). As such, although therapeutic potential of both ESCs and iPSCs is enormous due to their auto-reproducibility and pluripotentiality, there are still limitations to their practical use. For example, regulation of teratoma formation, ethical considerations when needing human cells, immune concerns regarding ESCs, and difficulties in genetic manipulation with respect to iPSCs (24). ASCs, as one of the important post-natal adult stem cell types, are one of the most promising stem cell populations identified thus far, as human adipose tissue is ubiquitous and easily obtained (after elective surgery) in large quantities with little donor site morbidity or patient discomfort. Thus, use of autologous ASCs as both research tools and as cell therapeutics is feasible, and has been shown to be both safe and efficacious in pre-clinical and clinical studies of injury and disease (25,26).

A number of articles have described secretory profiles of pre-adipocyte ASCs, determined mainly using enzyme-linked immunoabsorbent assays (27,28). In cultured ASCs at relatively early passages, secreted soluble factors such as VEGF, TGF- β_1 , IGF-1, basic FGF (bFGF), granulocyte-macrophage colony-stimulating factor, tumor necrosis factor (TNF)- α and interleukins have been confirmed (21,28). These released factors are believed to have potential in the early stages of vascular system formation. In this study, we screened growth factors and confirmed presence of enhanced ones in both ASCs and ECs after crosstalk between them, in angiogenesis, *in vitro*. Among these, increased *IGF-1*, *HIF-1 α* and *FGF-1/2* played roles of direct participation in vascular formation. In a previous report, Cao *et al.* demon-

strated significant neovascularization in an *in vivo* mouse hind limb ischemia model, after treatment with ASCs, and provided evidence to the effect that the ASCs could differentiate into endothelial cells (11). Considered together, it seems plausible that enhanced angiogenesis was supported not only by growth factors released from ASCs but also by direct participation of this cell type.

A further injection experiment concerning MSCs showed that the cells expressed angiogenic factors and induced host angiogenesis predictably through paracrine effects. Alternatively, these cells seemed to be partially incorporated into new vessels during the angiogenesis witnessed. Some transplanted MSCs expressed characteristic endothelial or smooth muscle cell proteins and incorporated into vascular networks within ischemic sites; however, frequency was significantly low as most cells died within the necessary low-mass transfer micro-environment (29,30). As it has also been shown that soluble factors could modulate ASCs in turn, it is promising that ASC function could be maintained and even improved using these enhanced growth factors.

BMPs also play an important role in angiogenesis. In cancer cells, BMPs can activate the VEGF promoter, and VEGF mRNA and protein expression have been shown in a prostate cancer cell line (31,32). In osteoblasts, BMP-2 and BMP-4 have been shown to stimulate angiogenesis by production of VEGFA (33) and in MSCs, BMP-2 has increased expression of placental growth factor (PIGA), a VEGF family member (34). BMP-5 has been shown to play important roles in sinusoidal endothelium of forming liver (35). BMP-7 is involved in improvement of recovery after ischemic insult, promoted endothelial cell survival and induced angiogenesis indirectly by increasing VEGF expression (36,37), while a BMP-7 variant (BMP-7v) has been shown to inhibit angiogenic endothelial cord formation in glioblastoma multiforme (38). In ASCs, a recent study has shown that angiogenic genes (*VE-cadherin*, *VEGFA* and *HIF-1 α*) were suppressed when treated with noggin or dorsomorphin, and as a result weakened angiogenesis (39–42). In our investigation, ASC co-culture promoted gene expressions of *BMP-5* and *BMP-7* in ECs. Increases could be synergistic enhancement of angiogenesis, following those of *IGF-1*, *HIF-1 α* and *FGF-1/2*.

It is necessary to mention some limitations of our study. First, angiogenesis in 3D gels involves cell–cell contact cross-talk. Here, to isolate gene expressions between ASCs and ECs, non-contact transwell co-culture was used. This could be inaccurate to some degree, due to gaps between contact and non-contact co-cultures. Secondly, we screened the growth factor profile

that was based on a common gene bank; other unscreened growth factors may also play vital roles in angiogenesis. Thirdly, gene and protein expressions by murine cells have many differences from those of human cells; results achieved from murine cells might not be applicable to human cells. Fourthly, assessment of expression of genes encoding growth factors was straightforward although various detection methods were necessary. Fifthly, exact mechanisms of ASC-promoting angiogenesis, through enhanced growth factors need to be further explored.

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Conflict of interests

The authors report no conflicts of interest and alone are responsible for content and writing of the article.

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