ORIGINAL ARTICLE

IGF-1 promotes angiogenesis in endothelial cells/adipose-derived stem cells co-culture system with activation of PI3K/Akt signal pathway

Shiyu Lin | Qi Zhang | Xiaoru Shao | Tao Zhang | Changyue Xue | Sirong Shi |
Dan Zhao | Yunfeng Lin

State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China

Correspondence

Yunfeng Lin, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China.
Email: yunfenglin@scu.edu.cn

Funding information

National Natural Science Foundation of China, Grant/Award Number: 81671031, 81470721 and 81621062; Sichuan Province Youth Science, Grant/Award Number: 2014TD0001

Abstract

Objectives: The aim of this study was to investigate the role of insulin-like growth factor-1 (IGF-1) and crosstalk between endothelial cells (ECs) and adipose-derived stem cells (ASCs) in the process of angiogenesis.

Methods: A three-dimensional collagen gel used to culture mouse ASCs and mouse ECs in vitro was established. The effects of angiogenesis after exposure to IGF-1 were observed by confocal laser scanning microscopy. Western blotting and qPCR were performed to elucidate the underlying mechanisms.

Results: IGF-1 treatment promoted the formation of vessel-like structures and the recruitment of ASCs in the three-dimensional collagen gel. The angiogenic genes and proteins in ECs were up-regulated by IGF-1 and in co-culture. Similar changes in the genes and in the proteins were detected in ASCs after exposure to IGF-1 and co-culture. p-Akt expression levels were high in ECs and ASCs after exposure to IGF-1 and co-culture.

Conclusions: IGF-1 and co-culture between cells facilitate the process of angiogenesis via the PI3-kinase/Akt signalling pathway. In ECs, IGF-1 stimulates the expression of angiogenesis-related growth factors with the activation of the PI3-kinase/Akt signalling pathway. Co-cultured ECs exposed to excess VEGF-A and other angiogenesis-related growth factors para-secreted from ASCs exhibit high expression of angiogenesis-related genes and proteins. In ASCs, IGF-1 induces the recruitment and function of ASCs by up-regulating the expression of *PDGFB*, *MMPs* and α -*SMA*. Crosstalk with ECs further facilitates changes in ASCs.

1 | INTRODUCTION

Angiogenesis plays a pivotal role in tissue regeneration and wound repair processes by providing oxygen and nutrients and eliminating metabolic products. Adequate tissue perfusion in the regenerating location plays a significant role in preserving viability during tissue regeneration by providing sufficient vascular beds. Inadequate

growth or maintenance of the vascular net may give rise to several types of morbidities, including myocardial infarction, ulceration in the skin or digestive tract, stroke and neurodegeneration in the nervous system.^{1,2} Angiogenesis is an intricate process involving the activation of vascular cells through a balance of pro- and anti-angiogenic factors.² It occurs through a sequence of procedures consisting of the recruitment of pericytes, stimulation of endothelial cells (ECs) by growth factors from the autocrine and paracrine systems, liberation of both the ECs and pericytes by degrading the

Shiyu Lin and Qi Zhang contributed equally to this work.

ambient extracellular matrix (ECM), EC proliferation and migration, and their ultimate reorganization into a three-dimensional tubular structure.³⁻⁵

Despite these known benefits, clinical trials on the administration of pro-angiogenic factors, including vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), have generally shown unsatisfactory results.⁶ Therefore, alternative strategies are needed to target cells other than ECs for indirectly regulating the process of angiogenesis.⁷

Cell-based vessel regeneration therapies have been applied as treatments for a series of ischaemic diseases, including myocardial infarction and peripheral vascular disease (PVD).⁸⁻¹⁰ The combined application of ECs and adipose-derived stem cells (ASCs) has proved to be a practical method for constructing stable vascular networks, with ASCs providing microvascular beds and up-regulating the secretion of bioactive factors.¹¹ Our previous studies demonstrated that few vessel-like structures were formed under mono-culture of ECs, although their numbers increased after co-culture with ASCs.^{12,13}

Insulin-like growth factor-1 (IGF-1) participates in several cell behaviours such as growth, differentiation and transformation, and has become a hot spot of research on various vascular diseases such as atherosclerosis, hypertension, angiogenesis and diabetic vascular disease.¹⁴ IGF-1 and its corresponding receptor can subsequently activate PI3K/Akt signalling pathways and further induce cellular actions such as proliferation, differentiation and migration.¹⁵ The interaction between IGF-1 and other growth factors further complicates IGF-1 signalling.¹⁶ In terms of angiogenesis, IGF-1 has a positive effect by exerting anti-apoptosis effects and promoting the migration of vascular smooth muscle cells. In addition, targeted expression of IGF-1 to the smooth muscle in transgenic mice led to smooth muscle hyperplasia in smooth muscle-rich tissues and significantly promoted the aortic medial area and thickness.¹⁷ Moreover, IGF-1 can promote glucose uptake, DNA synthesis, migration, inflammatory and vasodilatory responses, and angiogenesis in microvessel ECs.^{18,19}

The growth factor/PI3-kinase/Akt signalling pathway has been shown to play a key role in regulation of the angiogenic phenotype through the induction of angiogenic growth factors to stimulate PI3-kinase/Akt signalling in ECs in an autocrine manner.²⁰

Therefore, in the present study, we aimed to investigate the role of IGF-1 in an ECs/ASCs co-culture system with respect to angiogenesis and vascularization. Towards this end, a three-dimensional (3D) collagen gel was established containing fluorescently labelled ECs and ASCs, and the extent of microvessel formation was evaluated after exposure to IGF-1. We further explored the variations of relevant genes, proteins and signalling pathways to elucidate how IGF-1 and the crosstalk between ECs and ASCs influence the process of angiogenesis. Taken together, this work can help to highlight the changes in microvessel formation upon exposure to IGF-1, thereby paving the way towards new applications of IGF-1 and cell co-culture in vascular regeneration.

2 | METHODS

2.1 | Cell culture

The inguinal fat for ASCs was collected from 3-week female mice under aseptic conditions. The fat was cut finely and immersed in 0.075–0.1% collagenase I at 37°C in a water bath for 1 hour. Digestion was terminated with 10% FBS α -MEM and the suspension was centrifuged at 200 *g* for 5 minutes. Then the supernatant was removed and the remaining cells were resuspended with 10% FBS α -MEM. The suspension was inoculated into culture flasks and incubated at 37°C and 5% CO₂. ASCs were passaged to the third generation to obtain relatively pure ASCs.^{12,13}

We collected brain microvascular tissue from newborn mouse to obtain ECs. The samples were cut finely and digested with 0.5% collagenase II for 1 hour and then neutralized by the fresh high glucose DMEM with 10% FBS. After being centrifuged at 200 *g* for 8 minutes, the supernatant was removed and the remaining tissue was added with 20% BSA. After centrifugation at 1000 *g* for 20 minutes, the supernatant was removed and fresh 10% FBS DMEM was added to resuspend the ECs. The suspension was inoculated into plates and incubated at 37°C and 5% CO₂.¹²

Green fluorescent protein (GFP)-positive ASCs and DsRed-Express-positive ECs were harvested, respectively, from subcutaneous adipose tissue from enhanced GFP transgenic mice (The Centre of Genetically Engineered Mice, West China Hospital, Sichuan University, Chengdu, China) and brain microvascular tissues from DsRed-Express transgenic mice (The Genetic Centre of Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Centre of Comparative Medicine, Peking Union Medical College, Beijing, China).

TABLE 1 Primer sequences of GAPDH and target genes

Target gene (mouse)	Primer pairs
GAPDH	Forward: GGTGAAGTCCGGTGTGAACG Reverse: CTCGCTCTGGAAGATGGTG
VEGF-A	Forward: CTGCTGTGGACTTGTGTTGG Reverse: AAAGGACTTCGGCCTCTCTC
PDGFB	Forward: GGCTGTGACTTAGACAGGCT Reverse: TTTGAGTGAGACAGGCACCC
FGF-1	Forward: CCACAGCCCAGCAGTTATC Reverse: CTCCTACGCCCACTCTTCAG
TGF- β 1	Forward: TGGAGCCTGGACACACAGTA Reverse: TAGTAGACGATGGGCAGTGG
MMP-2	Forward: TCCTGCATCCACACAAAGAA Reverse: TTCCAGGGACACAGACATGA
MMP-9	Forward: ATGTGTCTTCCCCTTCACTTTC Reverse: GGTCATCATCGTAGTTGGTTGT
α -SMA	Forward: GCCAAGGTGACAGTGGGTAA Reverse: ATACTGGCTCAGGGGACTGT

2.2 | Co-culture system

In order to establish the three-dimensional collagen gel model, we used GFP-ASCs and RFP-ECs from transgenic mice. The two kinds of cells were suspended in a 1:1 ratio in HG-DMEM and rat tail muscle type I collagen (Shengyou Biotechnology, Hangzhou, China) to form a three-dimensional gel in 96-well plates. In the experimental group, 50 ng/mL IGF-1 (50 ng/mL; R&D Systems, Minneapolis, MN, USA) was added while PBS was added to the blank co-culture group. Cultures were incubated at 37°C for 7 days to form a gel sample. Morphology of vascular-like structures was observed by Olympus microscope, and images were analysed by Image Pro Plus 6.0.

Transwell co-culture was employed to explore gene and protein expressions of growth factors of ECs and ASCs after exposure to 50 ng/mL IGF-1 in 1% FBS DMEM and co-culture for 48 hours. ECs were seeded into 6-well plates with 2.5×10^5 cells per well and ASCs were seeded at 2×10^5 cells per well in transwell chambers.

Cell samples for gene and protein detections were divided into 3 groups: (i) mono-culture group, (ii) mono-culture group with IGF-1 and (iii) co-culture group with IGF-1.

2.3 | Quantitative PCR

Relative mRNA levels of VEGF-A, FGF-1, platelet-derived growth factor-B (PDGFB), transforming growth factor- β (TGF- β), α -smooth muscle actin (α -SMA), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) were investigated by qPCR. The sequences of primer pairs are listed in Table 1. Briefly, total RNA in ASCs and ECs was collected with RNeasy plus Mini Kit (Qiagen, Shanghai, China) after 48-hours exposure to IGF-1 and co-culture. A cDNA synthesis kit (MBI, Glen Burnie, MD, USA) was applied to prepare cDNA. Target cDNA was amplified as follow process: denaturation for 30 seconds at 95°C, followed by 5 seconds at 95°C and 31 seconds at 60°C for 40 cycles. The presence of primer dimers and false priming were detected by the melting curve.²¹

FIGURE 1 Insulin-like growth factor-1 (IGF-1) enhances the formation of vessel-like structures and recruitment of adipose-derived stem cells (ASCs). (A) Angiogenesis in 3D gel after 7 d of co-culture between ASCs and ECs with normal conditions and with IGF-1 in vitro. Results showed that co-culture IGF-1 significantly enhanced the formation of vessel-like structures ($n = 3$). Blue arrows indicate the connections and grey arrows indicate ASCs around ECs. (B, C) Analysis of the lengths and lumen diameters of vessel-like structures in both groups. (D) Analysis of the number of ASCs around ECs. The data were expressed as the mean of three different experiments ($n = 3$). Data were presented as means $\pm$ SD, * $P < .05$

2.4 | Western blot

Abundance of VEGF-A, MMP-2, MMP-9, α -SMA, total-Akt and p-Akt was examined by Western blot. ASCs and ECs were rinsed with cold PBS for 3 times and collected the total proteins with a whole cell lysis assay (KeyGen, Nanjing, China). The loading buffer and protein samples were mixed at a ratio of 1:4 and then boiled for 4 minutes. Subsequently, samples were separated by SDS-PAGE. And the related proteins were then transferred to the PVDF membrane. The membrane was soaked in 5% BSA for 1-hour blocking at room temperature. Then primary antibodies of VEGF-A (1 μ g/mL, ab51745; Abcam, Cambridge, UK), MMP-2 (1:3000, ab92536; Abcam), MMP-9 (1:500, ab58803; Abcam), α -SMA (1:200, ab5694; Abcam), total-Akt (1:2000, #29205; Cell Signaling Technology, Danvers, MA, USA) and p-Akt (1:2000, #4060; Cell Signaling Technology) were diluted with

5% BSA to the appropriate concentration and incubated overnight at 4°C. Relative secondary antibodies (Beyotime, Shanghai, China) were used to combine with primary antibodies for 1 hour. Subsequently, the bands were washed with TBST on the shaker for 3 times. Finally, ECL (enhanced chemiluminescence) detection system (Bio-Rad, Hercules, CA, USA) was employed for detection and the optical density method was employed to analyse the relative protein expression level.²²

2.5 | Statistical analysis

All experiments were performed independently in triplicate. And statistical analysis was performed with SPSS 19.0 (IBM, Silicon Valley, USA) using one-way ANOVA. Multiple comparisons were employed using Student-Newman-Keuls test or Dunnett's T3 test. Data were considered to be significantly different if the two-tailed *P*-value was <.05.

FIGURE 2 Gene profiles of angiogenesis-related growth factors in ECs treated with IGF-1 and co-culture. The gene variations of VEGF-A, TGF- β , PDGFB, FGF-1, MMP-2 and MMP-9 in mono-cultured ECs, mono-cultured ECs with IGF-1 and co-cultured ECs with IGF-1 were detected by qPCR. GAPDH levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD, * $P < .05$

3 | RESULTS

3.1 | Formation of vessel-like structures and recruitment of ASCs were enhanced by IGF-1 in a 3D collagen co-culture model

An in vitro 3D collagen co-culture model with RFP-mECs and GFP-mASCs was employed to determine whether IGF plays a role in angiogenesis. Based on microscope observations for 7 days after treatment, we observed more microvessel-like structures in the EC/ASC co-culture system supplemented with IGF-1 than in the co-culture group without IGF-1 (Figure 1A). In the co-culture group exposed to IGF-1, which exhibited more GFP-mASCs wrapping around, RFP-mECs proliferated, elongated and formed additional microvessel-like structures and connections (Figure 1A).

Subsequent analysis (using Image Pro Plus 6.0) of the lengths, widths and connections of vessels indicated a positive effect of IGF-1

on angiogenesis in the EC/ASC co-culture system. The number of microvessel-like structures and connections among RFP-mECs increased after exposure to IGF-1 (Figure 1A). Corresponding lengths and widths were longer than those in the control co-culture group (Figure 1B,C). As shown in Figure 1D, the number of ASCs in the region surrounding ECs increased after treatment with IGF-1.

3.2 | IGF-1 and co-culture influenced angiogenesis-related genes and proteins in ECs as well as the PI3K/Akt signalling pathway

According to our previous studies, co-culture of ECs and ASCs facilitates the formation of microvessels and enhances corresponding gene expression.¹¹ In our current study, exposure to IGF-1 promoted angiogenesis in the EC/ASC co-culture system (Figure 1); accordingly, we further explored the biological changes in the EC/ASC co-culture system.

FIGURE 3 Protein expressions of VEGF-A, MMP-2, MMP-9 and p-Akt in ECs were up-regulated by IGF-1 and crosstalk. Western blot showed that protein levels of VEGF-A, MMP-2 and MMP-9 in ECs exposed to IGF-1 and crosstalk were enhanced. Moreover, up-regulated p-Akt level was detected. GAPDH levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD, * $P < .05$

Transwell chambers were used for gene and protein detection. For this analysis, cell samples were divided into three groups: mono-cultured ECs, mono-cultured ECs with IGF-1 and co-cultured ECs with IGF-1.

The qPCR results (Figure 2A-F) for ECs indicated that the expression levels of *VEGF-A*, *TGF- β* , *PDGFB*, *FGF-1*, *MMP-2* and *MMP-9* in mono-cultured ECs were up-regulated after exposure to IGF-1. Moreover, *VEGF-A*, *PDGFB*, *FGF-1*, *MMP-2* and *MMP-9* were more highly expressed in co-cultured ECs treated with IGF-1 than in the other two groups. Western blotting was used to examine expression at the protein level for *VEGF-A*, *MMP-2* and *MMP-9*. As shown in Figure 3A and in the semi-quantitative analysis summarized in Figure 3B-D, we discovered that the trends in *VEGF-A* (increases of 1.77-fold and 2.82-fold higher in mono-cultured ECs with IGF-1 groups and mono-cultured ECs compared to mono-cultured ECs), *MMP-2* (increases of 4.03-fold and 7.24-fold higher in mono-cultured ECs with IGF-1 groups and mono-cultured ECs compared to mono-cultured ECs) and

MMP-9 (increases of 2.81- and 3.70-fold higher in mono-cultured ECs with IGF-1 groups and mono-cultured ECs compared to mono-cultured ECs) in ECs were similar to the results obtained at the level of gene expression. IGF-1 up-regulated the expression of angiogenesis-related genes and proteins in ECs. Moreover, crosstalk between ECs and ASCs amplified these effects.

To identify the underlying molecular mechanism, the PI3K/Akt signalling pathway in ECs was examined. The p-Akt level increased substantially after treatment with IGF-1 and co-culture (Figure 3E).

3.3 | Expression levels of angiogenesis-related genes and proteins in ASCs increased after IGF treatment and co-culture

In the EC/ASC co-culture system, ASCs act as pericytes and facilitate the expression of pericyte-related genes or angiogenesis-related genes,

FIGURE 4 Angiogenic growth factors in ASCs treated with IGF-1 and co-culture. The gene variations of *VEGF-A*, *TGF- β* , *α -SMA*, *FGF-1*, *MMP-2* and *MMP-9* in mono-cultured ASCs, mono-cultured ASCs with IGF-1 and co-cultured ASCs with IGF-1 were examined by qPCR. GAPDH levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD, * $P < .05$

further influencing ECs and thereby the angiogenesis process. Therefore, we investigated the molecular differences between mono-cultured ASCs, mono-cultured ASCs with IGF-1 and co-cultured ASCs with IGF-1. The up-regulation of angiogenesis-related genes was observed in ASCs exposed to IGF-1 and co-culture (Figure 4A-F). The expression levels of VEGF-A, TGF- β , FGF-1, α -SMA, MMP-2 and MMP-9 were up-regulated in mono-cultured ASCs treated with IGF-1. Furthermore, TGF- β , FGF-1, MMP-2 and MMP-9 were more highly expressed in co-cultured ASCs treated with IGF-1 than in mono-cultured ASCs treated with IGF-1. α -SMA, MMP-2, MMP-9 and VEGF-A were further examined at the protein level by Western blotting. As shown in Figure 5A,B, IGF-1 and co-culture synergistically up-regulated the expression of α -SMA (a pericyte marker²³), demonstrating that ASCs might function

as pericytes around ECs. Similar patterns were observed for the protein expression of MMP-2, MMP-9 and VEGF-A (Figure 5A,C,D,E).

The same analyses were applied to ASCs to investigate the potential signalling pathway involved in the tendency for ASCs to function as pericytes. Based on Western blot results, we found that p-Akt in ASCs (Figure 5F) was expressed at a high level after treatment with IGF-1 and co-culture.

4 | DISCUSSION

Cell-based vessel regeneration therapies are applied to ischaemic disorders, such as myocardial infarction and PVD.^{8,9,24} The combined

FIGURE 5 Protein expressions of VEGF-A, MMP-2, MMP-9 and p-Akt in ASCs were up-regulated by IGF-1 and crosstalk. Western blot showed that protein levels of VEGF-A, α -SMA, MMP-2 and MMP-9 in ASCs exposed to IGF-1 and crosstalk were enhanced. Similarly, up-regulated p-Akt level was detected. GAPDH levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD, * $P < .05$

application of ECs and ASCs has been established as a practical method to construct stable vascular networks; ASCs provide microvascular beds and up-regulate secreted bioactive factors.¹

IGF-1 is generated from cell types, such as vascular smooth muscle cells, macrophages and platelets, involved in atheroma formation.²⁵ In addition, IGF-1 expression is high at vascular injury sites, for example, in the walls of arteries with balloon denudation.²⁶

In our previous studies, we found that the co-culture of ECs and ASCs could facilitate the formation of microvessels and up-regulate the expression of angiogenesis-related genes.^{12,27,28} In the current study, we explored the role of IGF-1 in angiogenesis in the EC/ASC co-culture system and further investigated the mechanism of action of IGF-1 and underlying signalling pathways.

We report three main findings. (i) Exposure to IGF-1 facilitates the formation of microvessels and up-regulates the expression of angiogenesis-related genes and proteins in ECs and pericyte-related genes and proteins in ASCs. (ii) Co-culture increases the effect of IGF-1 on angiogenesis. (iii) The PI3K/Akt signalling pathway is involved in the process.

We propose a hypothesis for the regulatory effects of IGF-1 and EC/ASC co-culture on angiogenesis (Figure 6A). In ECs, IGF-1 stimulates the expression of angiogenesis-related growth factors. Co-cultured ECs

exposed to excess VEGF-A and other angiogenesis-related growth factors para-secreted from ASCs exhibit increased angiogenesis-related growth factor expression, including VEGF-A, PDGFB, TGF- β , FGF-1, MMP-2 and MMP-9.

As a significant regulator of physiological angiogenesis, VEGF-A promotes the growth of ECs in vitro and leads to an angiogenic response in vivo.²⁹ In addition to preventing apoptosis in ECs, VEGF-A plays a vital role in the mediation of vascular permeability and induction of endothelial fenestration in some vascular beds.³⁰ A series of cytokines, for example, TGF- β , PDGF and FGF, can up-regulate VEGF-A mRNA expression.³¹ Various growth factors, such as PDGFs, angiopoietins and TGF- β , are vital regulators in the process of proper vessel functioning by facilitating vessel maturation and the coverage of mural cells.^{31,32} To stabilize EC channels, PDGFBs are released from ECs to recruit PDGF receptor- β + pericytes.³³⁻³⁵ Mouse studies have demonstrated that the loss of the TGF- β receptors ALK-1, TGFR-1, TGFR-2 or ENG results in arteriovenous malformation.³⁶ As b-FGF can regulate adhesion and communication among cells via the regulation of cadherin, gap junction and integrin expression, it is vital for the maintenance of vascular integrity.^{37,38} During the process of vessel formation, MMP-2 and MMP-9 contribute to the degradation of the ECM, liberate ECs and pericytes at the surrounding site and convert

FIGURE 6 Illustrations of the regulation process of IGF-1 and crosstalk between ECs and ASCs. (A) Illustrations indicating that IGF-1 stimulates angiogenic growth factors in ECs and ASCs. Moreover, the effect of angiogenic growth factors was expanded with the existence of crosstalk between ECs and ASCs. (B, C) Illustrations showing the functions of related angiogenic growth factors in the process of angiogenesis

the characteristics of the basement membrane into a pro-angiogenic environment.³⁹ MMPs also liberate angiogenic factors, such as VEGF and FGF, from immobilized matrix stores.⁴⁰ The survival, proliferation, migration and vascular permeability of ECs are facilitated and the angiogenesis ability is reinforced.

The increased phosphorylation of Akt in ECs after treatment of IGF-1 indicated that the PI3-kinase/Akt signalling pathway was activated. Studies have shown that IGF-1 and its corresponding receptor can subsequently activate PI3K/Akt signalling pathways and further induce proliferation, differentiation and migration.¹⁵ The growth factor/PI3-kinase/Akt signalling pathway is a vital mediator of the angiogenic phenotype. Akt signalling is essential for the normal cellular G1/S phase transition and proliferation in ECs.^{41,42}

In ASCs, the up-regulation of α -SMA and MMPs indicates that IGF-1 induces the transition from ASCs to pericyte-like cells and establishment of the location around microvessels. In response to PDGFB from ECs, ASCs are recruited to the proximity of ECs via the degradation of the ECM with MMP-2/9, and then ASCs coat ECs to provide microvascular beds, functioning as pericyte-like cells by expressing smooth muscle proteins.

From the perspective of the process of angiogenesis, we summarized the angiogenic effects of IGF-1 and cell co-culture in Figure 6B,C. ECs proliferate, elongate and sprout to establish a neovessel (responding to increased VEGF-A, FGFs and MMPs^{29,30,37,38,40}). Proliferating ECs attract and recruit ASCs (via enhanced secretion of PDGFB and FGFs^{32-35,37,38}) and degrade the surrounding ECM (by high levels of MMP-2 and MMP-9 expression^{39,40}). After the fusion of neighbouring branches and the formation of vascular networks, the maturation of ASCs as pericytes (involving up-regulated VEGF and FGFs^{29,30,37,38}) and vascular maintenance (via enhanced secretion of PDGFB and TGF- β ^{32,36}) are required. All of these cytokines are up-regulated after exposure to IGF-1 and this up-regulation is further enhanced in EC/ASC co-culture. We have to admit that there are some limitations in our work. Firstly, in order to isolate the gene expressions in ECs and ASCs, non-contact transwell co-culture was used, while there were two forms of interactions between ECs and ASCs in 3D gel, including cell-cell direct contact and indirect contact (paracrine). Therefore, to have both profiles of gene expression—transwells versus cells in contact may better reveal the physiological mechanisms and we will carry it out in our next study. Secondly, we screened the growth factor profile which was based on the common genebank, and other unscreened growth factors may also play a vital role in angiogenesis.

ACKNOWLEDGEMENTS

The work was majorly supported by the National Natural Science Foundation of China (81671031, 81470721 and 81621062) and Sichuan Province Youth Science (2014TD0001).

ORCID

Yunfeng Lin <http://orcid.org/0000-0003-1224-6561>

REFERENCES

1. Traktuev DO, Prater DN, Merfeldt S, et al. Robust functional vascular network formation in vivo by cooperation of adipose progenitor and endothelial cells. *Circ Res*. 2009;104:1410.
2. Carmeliet PMJ. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 2000;6:389-395.
3. Liekens S, Clercq ED, Neyts J, Liekens S, De Clercq E, Neyts J. Angiogenesis: regulators and clinical applications. *Biochem Pharmacol*. 2001;61:253-270.
4. Gilbert S, Camara J, Camara R, et al. Contaminated open fracture and crush injury: a murine model. *Bone Res*. 2015;2:31-39.
5. Gong T, Xie J, Liao J, Zhang T, Lin S, Lin Y. Nanomaterials and regenerative medicine. *Bone Res*. 2015;3:15029.
6. Simons M. Angiogenesis: where do we stand now? *Circulation*. 2005;111:1556.
7. Carmeliet P. Review article: angiogenesis in life, disease and medicine. *Nature*. 2005;438:932-936.
8. Leistner DM, Fischerrasokat U, Honold J, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. *Clin Res Cardiol*. 2011;100:925.
9. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cells transplantation in humans. *Circulation*. 2002;106:1913-1918.
10. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*. 2002;360:427-435.
11. Bai Y, Wang W, Sun G, Zhang M, Dong J. Curcumin inhibits angiogenesis by up-regulation of microRNA-1275 and microRNA-1246: a promising therapy for treatment of corneal neovascularization. *Cell Proliferat*. 2016;49:751-762.
12. Cun X, Xie J, Lin S, et al. Gene profile of soluble growth factors involved in angiogenesis, in an adipose-derived stromal cell/endothelial cell co-culture, 3D gel model. *Cell Proliferat*. 2015;48:405.
13. Shi S, Xie J, Zhong J, et al. Effects of low oxygen tension on gene profile of soluble growth factors in co-cultured adipose-derived stromal cells and chondrocytes. *Cell Proliferat*. 2016;49:341.
14. Le RD. Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *New Engl J Med*. 1997;336:633.
15. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001;414:799.
16. Rao GN, Delafontaine P, Runge MS. Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-gamma 1 in rat aortic smooth muscle cells. *J Biol Chem*. 1995;270:27871.
17. Liao J, Cai X, Tian T, et al. The fabrication of biomimetic biphasic CAN-PAC hydrogel with a seamless interfacial layer applied in osteochondral defect repair. *Bone Res*. 2017;5:17018.
18. Kondo T, Vicent D, Suzuma K, et al. Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization. *J Clin Invest*. 2003;111:1835-1842.
19. Zhang M, Sheng Y. Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels. *Arterioscler Thromb Vas*. 2004;24:435.
20. Lau MT, Leung PC. The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells. *Cancer Lett*. 2012;326:191-198.
21. Zhang T, Gong T, Xie J, et al. Softening substrates promote chondrocytes phenotype via RhoA/ROCK pathway. *Acs Appl Mater Inter*. 2016;8:22884.
22. Shao X, Lin S, Peng Q, et al. DNA nanostructures: tetrahedral DNA nanostructure: a potential promoter for cartilage tissue regeneration

- via regulating chondrocyte phenotype and proliferation. *Small*. 2017;13:1602770.
23. Kutluk CB, Ostapoff KT, Gerber DE, et al. BIBF 1120 (Nintedanib), a triple angiokinase inhibitor, induces hypoxia but not EMT and blocks progression of preclinical models of lung and pancreatic cancer. *Mol Cancer Ther*. 2013;12:992-1001.
 24. Karpov AA, Udaloova DV, Pliss MG, et al. Can the outcomes of mesenchymal stem cell-based therapy for myocardial infarction be improved? Providing weapons and armour to cells. *Cell Proliferat*. 2017;50:e12316.
 25. Bornfeldt KE, Arnqvist HJ, Norstedt G. Regulation of insulin-like growth factor-I gene expression by growth factors in cultured vascular smooth muscle cells. *J Endocr*. 1990;125:381-386.
 26. Cercek B, Fishbein MC, Forrester JS, Helfant RH, Fagin JA. Induction of insulin-like growth factor I messenger RNA in rat aorta after balloon denudation. *Circ Res*. 1990;66:1755-1760.
 27. Zhao D, Xue C, Lin S, et al. Notch signaling pathway regulates angiogenesis via endothelial cell in 3D co-culture model. *J Cell Physiol*. 2017;232:1548-1558.
 28. Zhong J, Guo B, Jing X, et al. Crosstalk between adipose-derived stem cells and chondrocytes: when growth factors matter. *Bone Res*. 2015;4:15036.
 29. Ferrara N. *The Biology of Vascular Endothelial Growth Factor*. *Endocrine Reviews*. 1997; 18:4.
 30. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-1309.
 31. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res*. 2005;97:512-523.
 32. Saik JE, Gould DJ, Watkins EM, Dickinson ME, West JL. Covalently immobilized platelet-derived growth factor-BB promotes angiogenesis in biomimetic poly(ethylene glycol) hydrogels. *Acta Biomater*. 2011;7:133.
 33. Tirziu D, Jaba I, Moodie KL, et al. Abstract 12382: Endothelial specific inhibition of NF-kappa B results in reduced vessel maturation due to poor monocyte recruitment and impaired notch and PDGF signaling. *Circulation*. 2010;122:A12382.
 34. Gaengel K, Genove GA, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscl Thromb Vas*. 2009;29:630-638.
 35. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298-307.
 36. Pardali E, Goumans MJ, Ten DP. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol*. 2010;20:556-567.
 37. Cao Y, Cao R, Hedlund EM. R Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways. *J Mol Med*. 2008;86:785-789.
 38. Murakami M, Nguyen LT, Zhuang ZW, et al. The FGF system has a key role in regulating vascular integrity. *J Clin Invest*. 2008;118:3355.
 39. Schultz GS, Wysocki A. Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen*. 2009;17:153.
 40. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol*. 2000;2:737.
 41. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci USA*. 2000;97:1749.
 42. Jiang BH, Liu LZ. AKT signaling in regulating angiogenesis. *Curr Cancer Drug Tar*. 2008;8:19.

How to cite this article: Lin S, Zhang Q, Shao X, et al. IGF-1 promotes angiogenesis in endothelial cells/adipose-derived stem cells co-culture system with activation of PI3K/Akt signal pathway. *Cell Prolif*. 2017;50:e12390.
<https://doi.org/10.1111/cpr.12390>