Effects of low oxygen tension on gene profile of soluble growth factors in co-cultured adipose-derived stromal cells and chondrocytes

Sirong Shi, Jing Xie, Juan Zhong, Shiyu Lin, Tao Zhang, Ke Sun, Na Fu, Xiaoru Shao and Yunfeng Lin

State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

Received 22 October 2015; revision accepted 28 March 2016

Abstract

Objectives: Moving towards development of optimized cartilage regeneration with adipose-derived stromal cells (ASCs), the focus of this study was on investigating the influence of hypoxia on soluble factors secreted by ASCs and chondrocytes after crosstalk.

Methods: We established direct contact co-culture and non-contact co-culture systems by using red or green fluorescent protein (R/GFP)-labelled mice and SD rats respectively. Gene variation of growth factors of the two cell types, in both hypoxic and normoxic conditions, were screened using semi-quantitative polymerase chain reaction (PCR).

Results: Co-culture with ASCs and chondrocytes under hypoxia was shown to successfully induce or enhance ASC to chondrogenic differentiation. To be specific, chondrogenic marker genes: *AGC*, *COL II* and *SOX9* were remarkably enhanced in both ASCs and chondrocytes after crosstalk under low oxygen tension. Subsequently, screening growth factors in ASCs and chondrocytes under hypoxia showed that *HIF-1 α* , *VEGF-A/B*, *BMP-2/-4/-6*, *FGF-2* and *IGF-1* were significantly increased, but not *TGF- β 1*.

Conclusions: These results revealed that both hypoxia and co-culture systems can notably enhance chondrogenesis of ASCs as well as increase proliferation of ASCs and chondrocytes.

Abbreviations

ACI: autologous chondrocytes implantation

AGC: Aggrecan

ASCs: adipose-derived stromal cells

BMP (-2, -4, -5, -6 and -7): bone morphogenetic protein (-2, -4, -5, -6, and -7)

COL II: type-II collagen

DMEM: Dulbecco's modified Eagle media

ELISA: enzyme-linked immunosorbent assay

FBS: foetal bovine serum

FGF-2: fibroblast growth factor-2

GAG: glycosaminoglycan

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HGF: hepatocyte growth factor

HIF-1 α : hypoxia-inducible factor-1 α

IGF-1: insulin-like growth factor-1

MSCs: mesenchymal stromal cells

OA: osteoarthritis

PCR: polymerase chain reaction

PTHrP: parathyroid hormone-related peptide

qPCR: quantitative polymerase chain reaction

R/GFP: red or green fluorescent protein

SOX9: Sry-containing box gene 9

TGF- β 1: transforming growth factor- β 1

VEGF-A/B: vascular endothelial growth factor A/B

α -MEM: α -glucose Dulbecco's modified Eagle's media

β -actin: beta-actin

Correspondence: Yunfeng Lin, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China. Tel.: +86 28 85503487; Fax: +86 28 85582167; E-mail: yunfenglin@scu.edu.cn
Sirong Shi and Jing Xie contributed equally to this work.

Introduction

In the physiological condition, it is generally known that articular cartilage, an avascular tissue, derives both

its nutrition and oxygen supply by diffusion from the synovial fluid and the subchondral bone, functioning at lower oxygen tension than that of most other tissues (1–4). Previous studies demonstrated that the oxygen tension in the chondrocytes at the articular surface is approximately 6–10% O₂, in the deepest bone layers having access to no more than 1–6% O₂ (5–7). Articular cartilage, once injured by trauma or pathology, has an extremely limited capacity to heal by itself. In orthopaedic surgeons, it is still a significant challenging problem to cure of focal or dispersed cartilage defects (8). Cartilage tissue engineering poses extraordinarily potential to alleviate cartilage-related diseases. The greatly promising healing therapy, autologous chondrocytes implantation (ACI), which is used to treat cartilage defects still has disadvantages such as limited sources of chondrocytes and high donor site morbidity (9,10).

Adipose-derived stromal cells (ASCs), naturally residing in adipose tissue, are capable of self-renew and can differentiate into chondrocytes, osteoblasts and adipocytes (11). Compared to bone marrow-derived mesenchymal stromal cells (MSCs), ASCs possess not only proliferation, long-term self-renewal and multilineage differentiation but also accessibility and abundance. It can be easily obtained from abdominal fat or infrapatellar fat by minimally invasive techniques and harvested a large number of cells (12–14). These significantly practical superiorities make ASCs an extremely dramatic cell population for applying to cartilage repair. Chondrogenic differentiation of ASCs may be induced by specific growth and transcriptional factors (15), provision of suitable three-dimensional (3-D) environment (16) and co-culture techniques (17,18). In our previous study, co-culture ASCs and chondrocytes seeded on the 3D P3HB4HB scaffolds was implanted in cartilage defects, stimulating cartilage formation (19). Nevertheless, the potential influence of soluble growth factors between ASCs and chondrocytes remains to be elaborately elucidated.

Several studies indicate that chondrogenic differentiation of MSCs could be highly enhanced under low oxygen tension (20–22). Interestingly, Merceron *et al.* found that 5% O₂ promotes the chondrogenesis of ASCs (23). Subsequently, an increasing number of studies demonstrate that treatment with hypoxia of ASCs not only merely maintains their morphology and surface markers but also augments the expression of stemness marker and proliferation, especially strengthening the chondrogenic differentiation capacity (24,25). As mentioned above, the microenvironment of articular chondrocytes is low oxygen tension. Taken together, hypoxia is supposed to be a significant factor in both maintaining chondrocytes phenotype and cementing ASCs chondrogenesis. It has been reported that

hypoxia-inducible factor-1 α (*HIF-1 α*) plays a fatal role in chondrogenesis, mediating the transcriptional response to hypoxia (26). Meanwhile, vascular endothelial growth factor (*VEGF*), promoting in angiogenesis in physiological low oxygen tension, can mediate normal chondrocytes differentiation (27,28). However, there still remains indistinct about how low oxygen environment mediates the chondrogenesis of ASCs co-culture with chondrocytes. Additionally, the genetic expression levels of the potential soluble growth factors secreted by ASCs and chondrocytes under physiological oxygen tension after co-culture are yet unknown.

Therefore, in our present study, we investigated the effect of low oxygen tension on the average soluble growth factors after crosstalk between ASCs and chondrocytes by means of the co-culture system treated with hypoxia condition. We mainly detected the expression of relevant growth factors in order to determine the vital ones participating in facilitating chondrogenesis, and offer a direct clue paracrine effects on the cartilage repair and regeneration.

Material 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).

Adipose-derived stromal cells were obtained, isolated and harvested from subcutaneous adipose tissue of 5-day SD female rats. Briefly, collected adipose tissue was cut into small pieces and digested with 0.75% type I collagenase at 37 °C with vigorous agitation, for 30 min. Enzyme activity was neutralized 1:1 (v/v) with fresh α -glucose Dulbecco's modified Eagle's media (α -MEM; Hyclone, Logan, UT, USA) containing 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Hyclone, Logan, UT, USA). The mixed suspension was centrifuged at 200 *g* for 5 min. After removing the first supernatant, 10% FBS α -MEM was added to centrifuge tubes to re-suspend the ASCs. Subsequently, the ASCs in suspension were seeded into T25 culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO₂ until use. Purified ASCs could then be obtained after two passages as described previously (29).

The primary rat articular chondrocytes were isolated from newborn SD rats about 3-day old. In brief, under sterile conditions, cartilage tissues derived from the exposed knee joint was minced into small pieces, pre-treated with 0.25% trypsin for 30 min, washed three times with phosphate-buffered saline (PBS) to eliminate

the trypsin, and digested with type-II collagenase (0.1%) for 2–3 h in 37 °C water bath. The chondrocytes suspension was collected and mixed 1:1 (v/v) with fresh Dulbecco's modified Eagle media (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin solution. After centrifuged at 200 g for 5 min, removing the supernatant, the isolated chondrocytes were resuspended in 10% FBS DMEM and seeded on T25 culture flasks and then cultured at 37 °C in a humidified atmosphere of 5% CO₂ atmosphere till passage II for usage.

To obtain green fluorescent protein (GFP)-positive ASCs and DsRed-Express-positive chondrocytes, the subcutaneous adipose tissue and knee joint were collected from enhanced GFP transgenic mice (The Centre of Genetically Engineered Mice, West China Hospital, Sichuan University, Chengdu, China) and the 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) respectively. Cell isolation for these was as described above.

Co-culture system

Obtaining for the observation of cell morphology, we adopted the cell–cell contact co-culture between GFP-ASCs and RFP-chondrocytes to see the visualized cell morphologies, and the cell–cell non-contact co-culture between ASCs and chondrocytes to see the change of different cell skeletons. GFP-ASCs and RFP-chondrocytes were mixed at a 1:1 ratio, seeded into six-well plates at 37 °C and cultured for 1 week. After mono-culture or co-culture for 3, 5 and 7 days in different oxygen conditions, cell morphologies were observed by fluorescence microscope. The ASCs or chondrocytes were seeded in six-well plates, and simultaneously, the ASCs or chondrocytes were also implanted onto transwell at 1×10^4 cells/cm² (six-well plates, Corning, Jiangsu, China) with 0.4 µm porous membrane. ASCs or chondrocytes were cultured in the 10%FBS α -MEM media and 10%FBS DMEM, respectively. After the cells were adherent to plates or inserts, the culture media were replaced with 10% FBS media for a 12 h equilibration, changed by 2% FBS DMEM for a 8 h starvation, decreased in FBS at 1% for co-culturing ASCs with chondrocytes in different oxygen concentrations of 21% and 2% respectively. Immunofluorescent staining observes the cell morphologies and skeletons by confocal laser scanning microscopy (CLSM) on the third day. For detecting the expression of genes, the gene profile of soluble growth factors in ASCs and chondrocytes was detected using transwell co-culture system as described above. At 1, 2, 3, 5 and 7 days after

incubation in 21% and 2% O₂, respectively, cell lysate samples (1000 µl) were collected for semi-quantitative polymerase chain reaction.

Immunofluorescent staining

After 3 days of incubation about mono-culture and co-culture in 21% and 2% O₂, respectively, abandoning the transwell inserts, the cells (ASCs or chondrocytes) seeded in six-well plates were washed with PBS three times, fixed with 4% paraformaldehyde for 20 min, followed by rinsing three times with PBS, and treated with 0.5% Triton X-100 in PBS for 15 min. Cleaning three times with PBS, the cells were incubated with FITC-phalloidine with 1:100 dilutions. The samples were then stained with DAPI for 10 min, and images were captured with the CLSM.

Semi-quantitative polymerase chain reaction

The RNA samples of ASCs and chondrocytes were collected, isolated and purified using the RNeasy Plus Mini Kit (Qiagen, CA, USA) with genomic DNA eliminator, dissolved in the RNase-free water, and quantified by measuring absorbance at 260 nm using a spectrophotometer. Dissolved RNA samples were then treated with DNase I (Mbi, Glen Burnie, MD, USA). Preparing cDNA of each sample uses cDNA synthesis kit (Mbi, Glen Burnie, MD, USA) with a final volume of 20 µl.

To assess the mRNA expression levels of growth factors in all samples as normalized to housekeeper genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (β -actin), semi-quantitative PCR was accomplished with a PCR kit (Mbi, Glen Burnie, MD, USA) in a 25 µl volume containing a 1 µl cDNA sample using a thermo-cycler (Bio-Rad, Hercules, CA, USA). Sets of primers chosen were displayed in the Table 1. The BLAST was used to search for all primer sequences to ensure gene specificity. The procedure of PCR as follows: cDNA was denatured for 30 s at 94 °C, 30 s annealing cycle at 55–65 °C and 72 °C, 30 s elongation cycle, 22–28 amplification cycles. The 2% agarose gel electrophoresis resolved in TAE buffer were used to visualize bands which represented the expressing dose of the soluble growth factor genes by staining with ethidium bromide. The data quantification was achieved by optical density with Image-Pro Plus 6.0. (Media Cybernetics, Rockville, MD, USA)

Western blot analysis

Cells were washed three times with ice-cold PBS, then harvested and lysed in lysis buffer containing protease

Table 1. The primer sequences of housekeeper genes and related soluble growth factor genes designed for semi-quantitative PCR

mRNA	Product length	Primer pairs	
GAPDH	233 bp	Forward	ACAGCAACAGGGTGGTGGAC
		Reverse	TTTGAGGGTGCAGCGAACTT
β -ACTIN	266 bp	Forward	CACCCGCGAGTACAACCTTC
		Reverse	CCCATACCCACCATCACACC
HIF-1 α	122 bp	Forward	CGATGACACGAACTGAAG
		Reverse	CAGATTCAAGTAATGGAGACA
COL II	116 bp	Forward	TCAAGTCGCTGAACAACCAG
		Reverse	G TCTCCGCTCTTCCACTCTG
Aggrecan	137 bp	Forward	GCAGCACAGACACTTCAGGA
		Reverse	CCCCTTTCTACAGGCAAGC
SOX9	120 bp	Forward	TTGGTCCGAGGTCTCTAAGGT
		Reverse	AAAGTTGTCGCTCCCACTGA
BMP-2	102 bp	Forward	TCAAGCCAAACACAAACAGC
		Reverse	CCACGATCCAGTCATTCCA
BMP-4	101 bp	Forward	GACTTCGAGGCGACACTTCT
		Reverse	AGCCGGTAAAGATCCCTCAT
BMP-6	101 bp	Forward	TGTCAGAGGGAGAGGGACTG
		Reverse	CTTGCGGTTCAGGGAGTGT
VEGF-A	154 bp	Forward	TCATCAGCCAGGGAGTCTGT
		Reverse	TGAGGGAGTGAAGGAGCAAC
VEGF-B	127 bp	Forward	GCAACACCAAGTCCGAATG
		Reverse	TGGCTTCACAGCACTCTCC
TGF- β_1	204 bp	Forward	CCGCAACAACGCAATCTAT
		Reverse	CCAAGGTAACGCCAGGAAT
FGF-2	106 bp	Forward	CCATCAAGGGAGTGTGTGC
		Reverse	TCCAGGCGTTCAAAGAAGAA
IGF-1	106 bp	Forward	TCTACCTGGCACTCTGCTTG
		Reverse	GGTCCACACACGAACTGAAG

inhibitors. The lysates were centrifuged at 10310 g for 5 min at 4 °C. The supernatant was collected, and the protein concentrations were determined by bicinchoninic acid (BCA) assay. Protein samples were solubilized and boiled in SDS sample buffer for 5 min and then separated using 10% or 6% SDS-PAGE at 100V for 90 min. Subsequently, the separated proteins were transferred to a polyvinylidene difluoride membrane. Following incubation in blocking solution consisting of 5% BSA in TBST for 1 h at room temperature and overnight incubation at 4 °C with the primary antibodies including *GAPDH*, *SOX9*, *COL II* and *AGC*, the membrane was washed and then probed with respective secondary antibodies for 1 h at room temperature. After washing three times with TBST, hybridization was visualized using the ECL chemiluminescence detection system. Expression levels of the proteins were compared to the control based on the relative intensities of the bands.

ELISA

COL II and *AGC* produced in supernatant media under hypoxia or normoxia in co-cultured ASCs and in

Table 2. Gene changes of chondrogenesis-related soluble growth factors under hypoxia or normoxia in mono-cultured or co-cultured ASCs and chondrocytes

Gene profile of growth factors	Mean of fold value (ratio to normal ASCs)				
	Co-culture		Hypoxia		Combined effect
(a)					
Aggrecan	0.797	-	0.933	-	0.841 -
COL-2	0.415	↓*	3.424	↑*	0.456 ↓*
SOX-9	0.525	-	1.650	↑*	5.027 -
HIF-1 α	1.652	↑*	1.830	↑*	2.223 -
VEGF-A	0.289	↓*	0.401	↓*	0.461 -
VEGF-B	1.522	↑*	1.333	↑*	1.599 -
BMP-2	1.743	↑*	0.717	-	1.166 -
BMP-4	0.920	-	0.460	↓**	0.437 -
BMP-6	0.654	-	1.129	-	0.959 -
IGF-1	1.056	-	2.001	↑**	2.219 -
FGF-2	0.976	-	2.826	↑*	1.589 -
TGF- β_1	0.760	↓*	0.650	↓***	0.189 -
Gene profile of growth factors	Mean of fold value (ratio to normal chondrocytes)				
	Co-culture		Hypoxia		Combined effect
(b)					
Aggrecan	3.190	↑*	1.862	↑*	1.808 -
COL-2	0.593	↓*	0.718	-	3.409 -
SOX-9	0.514	-	1.651	↑*	1.120 -
HIF-1 α	1.922	↑*	0.723	-	0.948 -
VEGF-A	0.963	-	0.804	-	1.784 -
VEGF-B	0.982	-	0.811	-	1.399 -
BMP-2	1.638	↑*	2.340	↑*	2.146 -
BMP-4	1.347	↑*	1.551	↑**	1.862 -
BMP-6	0.666	↓*	0.760	↓*	3.592 ↑*
IGF-1	0.848	-	1.679	↑*	1.955 -
FGF-2	0.999	-	2.536	↑*	1.201 -
TGF- β_1	0.911	-	1.131	-	1.061 -

The fold values were calculated by OD method with Quantity One 4.6.3 software (Bio-Rad, Hercules, CA, USA) based on the semi-quantitative PCR [mean of fold value represented the mean average ratio of experimental group to control group (mathematical average of all time points in every group)] ($n = 5$). Co-culture (vertical lane) denotes ratios of normoxia co-culture group with respect to normoxia mono-culture group; hypoxia (vertical lane) denotes ratios of hypoxia mono-culture group with respect to normoxia mono-culture group; combined effect (vertical lane) denotes ratios of hypoxic co-culture group with respect to normoxia mono-culture group; ↓ or ↑ means trend of fold change. *Significant difference with respect to normal control, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, '-' denotes no statistical differences.

co-cultured chondrocytes on day 5 were detected by ELISA according to the manufacturer's protocols. ELISA kits used were rat *COL II* ELISA kit (Cloud-clone corp. SEA572Ra) and rat *AGC* ELISA kit (Cloud-clone corp. SEB908Ra). Optical density was determined

using a microplate reader with 450 nm wave length. All experiments were carried out in triplicate.

Statistical analysis

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

Results

Different morphological features under hypoxia in co-cultured ASCs and chondrocytes

After 3 days co-culture in different oxygen conditions, cell morphologies of ASCs and chondrocytes were observed by fluorescence microscope (Fig. 1b) and CLSM (Fig. 1c). There was a significant increase in cell numbers of both ASCs and chondrocytes under low oxygen tension, which may demonstrate that hypoxia promoted the proliferation of ASCs and chondrocytes. The results of CLSM showed that ASCs morphologies had an

increasing tendency to differentiate chondrocytes under hypoxia after crosstalk, and the fibrosis of chondrocytes was decreased in the condition of physiological low oxygen tension. Additionally, under hypoxia, the proliferation of ASCs and chondrocytes enhanced no matter in the mono-culture groups or the co-culture groups (Fig. 1d).

The expressions of chondrogenic specific markers enhanced under hypoxia in co-cultured ASCs

We investigated the expression of *AGC*, *Col II* and *SOX9* genes of ASCs and chondrocytes both in normoxia and hypoxia detected at 1, 2, 3, 5 and 7 days and the expression of *AGC*, *Col II* and *SOX9* proteins on day 5 respectively. Results show that hypoxia up-regulates the *AGC*, *Col II* and *SOX9* genes both in ASCs and chondrocytes not only mono-culture but also co-culture (Fig. 2). Meanwhile, the expression of these three proteins also enhanced under hypoxia in co-cultured ASCs (Fig. 3a) and in co-cultured chondrocytes (Fig. 3b). Under hypoxia in co-cultured ASCs, the three marker genes in chondrocytes expressed a higher level when incubated with ASCs (*Col II* was as high as 3.41-fold, *AGC* was 1.81-fold and *SOX9* was 1.12-fold (Table 2)), growth of time, particularly at 5 and 7 days (Fig. 2b). Specifically, *SOX9* obviously strengthened

Figure 1. Different morphological features under hypoxia in co-cultured ASCs and chondrocytes. (a) The cell morphologies of ASCs and chondrocytes under hypoxia after crosstalk. (b) Representative cell morphologies in co-culture using ASCs from GFP-labelled mice and chondrocytes from RFP-labelled mice. (c) Cytoskeleton staining of ASCs and chondrocytes after 3 days' mono- and co-culture under normoxia and hypoxia. (d) Analysis of the proliferation in co-cultured ASCs and in co-cultured chondrocytes under hypoxia or normoxia, using Image-Pro Plus Software 6.0 (Media Cybernetics, Rockville, MD, USA).

Figure 2. The marker genes of chondrocytes enhanced under hypoxia in co-cultured ASCs. The gene expressions of *aggrecan*, *COL II* and *SOX9* under hypoxia or normoxia in co-cultured ASCs (a) and in co-cultured chondrocytes (b). *GAPDH* and β -*ACTIN* were used as internal references. The product sizes were indicated in the left lane. The samples were collected at 1, 2, 3, 5 and 7 days. The gels shown were representative of three different experiments ($n = 3$).

Figure 3. The expressions of chondrogenic-specific markers detected by WB and ELISA under hypoxia in co-cultured ASCs and in co-cultured chondrocytes. The protein expressions of *SOX-9*, *COL II* and *AGC* under hypoxia or normoxia in co-cultured ASCs (a) and in co-cultured chondrocytes (b) were measured by Western blotting. *GAPDH* was used as internal reference. The gels shown were representative of three different experiments ($n = 3$). The results of ELISA analysis for *COL II* (c) and *AGC* (d) in supernatant media under hypoxia or normoxia in co-cultured ASCs and in co-cultured chondrocytes on day 5. The data are represented as the mean $\pm$ SD of at least three independent experiments. Statistical analysis: *** $P < 0.001$.

under hypoxia in co-cultured ASCs. Moreover, its expression increased in a time-dependent manner, especially at 5 and 7 days (up to 5.03-fold) (Fig. 2a). As illustrated in Fig. 3a and 3b, not only merely hypoxia but also co-culture promotes the expressions of *SOX9*, *Col II* and *AGC* in both ASCs and chondrocytes. Not coincidentally, the results of ELISA about *Col II* (Fig. 3c) and *AGC* (Fig. 3d) indicate that hypoxia significantly enhanced the production of *Col II* and *AGC*. In general, *Col II* production under hypoxia is much higher in comparison to normoxia (** $P < 0.001$) both

in co-cultured ASCs and in co-cultured chondrocytes. Moreover, there is a remarkable difference (** $P < 0.001$) between hypoxia and normoxia both in co-cultured ASCs and in co-cultured chondrocytes about *AGC*.

Hypoxia modulates relevant growth factors in co-cultured ASCs and chondrocytes

Angiogenesis-related factors including *HIF-1 α* , *VEGF-A* and *VEGF-B* were investigated in our current study. For

both ASCs and chondrocytes, the expression of *HIF-1 α* significantly increased in the low oxygen tension (especially ASCs was up to 2.22-fold (Table 2)) (Fig. 4). No matter in hypoxia or normoxia, co-culture system has a stronger effect than mono-culture, the former showing a higher expression of *HIF-1 α* than the latter. *VEGF-A* and *VEGF-B* were up-regulated in hypoxia condition in both ASCs and chondrocytes (Fig. 4). *VEGF-A* increased at 5 and 7 days under hypoxia in co-cultured ASCs (up to 1.78-fold in chondrocytes (Table 2)) (Fig. 4a). Likewise, under low oxygen tension, *VEGF-B* expression in the co-culture group was higher than in the mono-culture group, in ASCs increasing to 1.60-fold (Fig. 4a), and while *VEGF-B* in chondrocytes was increased to 1.40-fold (Fig. 4b).

Other soluble growth factors related to chondrogenesis, including *IGF-1*, *FGF-2* and *TGF- β 1*, were investigated in both ASCs and chondrocytes. The expression level of *IGF-1* was increased in low oxygen tension in co-cultured ASCs (up to 2.22-fold (Table 2)) and chondrocytes (up to 1.96-fold) (Fig. 4). Compared to normoxia, *TGF- β 1* showed a little decrease in hypoxia, likewise, co-culture also down-regulated the expression of *TGF- β 1* (down to 76% in ASCs and down to 91.1% in chondrocytes) (Fig. 4). As to *FGF-2*, hypoxia promoted its expression in co-cultured and mono-cultured ASCs relative to normoxia. Additionally, in hypoxia and normoxia condition, co-culture down-regulated its expression in relative to mono-culture (Fig. 4a). In chondrocytes, no significant differences were found in *FGF-2* expression (Fig. 4b).

BMP family was modulated under hypoxia in co-cultured ASCs and chondrocytes

Bone morphogenetic protein (*BMP*) family, synthesized and secreted by osteoblasts, is mainly responsible to induce bone formation, including *BMP-2*, *BMP-4*, *BMP-5*, *BMP-6* and *BMP-7*. In ASCs, *BMP-2* expression in the co-culture system enhanced compared to that in the mono-culture group (up to 1.17-fold (Table 2)), while showing no difference between hypoxia and normoxia. The expression level of *BMP-4* is detectable, but no change in all groups. *BMP-6* increased under hypoxia in both mono-culture and co-culture groups compared with that under normoxia (up to 1.13-fold (Table 2)) (Fig. 5a). In chondrocytes, *BMP-2* expression increased in the co-culture system compared to the mono-cultures in the low oxygen tension (up to 2.15-fold (Table 2)). Noteworthy differences of *BMP-4* were detected in hypoxia in contrast with normoxia in co-cultured ASCs, hypoxia enhancing its expression up to 1.86-fold in co-cultured ASCs. *BMP-6* was obviously increased at 5 and 7 days under hypoxia in co-cultured ASCs (up to 3.59-fold (Table 2)) (Fig. 5b). *BMP-5* and *BMP-7* were also detected in both ASCs and chondrocytes but no variation (data not shown).

Discussion

In recent years, an increasing number of studies have demonstrated that chondrogenic differentiation could be induced by hypoxia condition or co-culture system,

Figure 4. Hypoxia modulates relevant growth factors in co-cultured ASCs and chondrocytes. The gene expressions of *HIF-1 α* , *VEGF-A*, *VEGF-B*, *IGF-1*, *FGF-2* and *TGF- β 1* under hypoxia or normoxia in co-cultured ASCs (a) and in co-cultured chondrocytes (b). *GAPDH* and β -*ACTIN* were used as reference genes. The product sizes were indicated in the left lane. The samples were taken at 1, 2, 3, 5 and 7 days. The gels shown were representative of three different experiments ($n = 3$).

Figure 5. BMP family was modulated under hypoxia in co-cultured ASCs and chondrocytes. The gene expressions of BMPs (BMP-2, BMP-4 and BMP-6) under hypoxia or normoxia in co-cultured ASCs (a) and in co-cultured chondrocytes (b). *GAPDH* and β -*ACTIN* were used as reference genes. The product sizes were indicated in the left lane. The samples were taken at 1, 2, 3, 5 and 7 days. The gels shown were representative of three different experiments ($n = 3$).

whereas the effect of combining the two approaches on chondrogenesis still remains unclear. Therefore, in our present study, we investigated a multiple gene profile of soluble growth factors, related to hypoxia, cell differentiation and chondrogenesis, secreted by both ASCs and chondrocytes after crosstalk under different oxygen tension including normoxia (21%) and hypoxia (2%).

Adipose-derived stromal cells, being considered a more convenient acquisition source of cells and promising candidate than MSCs, can be differentiated into chondrocytes, osteoblasts and adipocytes; hence, it has been extensively applied in tissue engineering (11). Recent progress indicated that the co-culture between MSCs and chondrocytes promoted the differentiation of MSCs towards chondrocytes. Chondrogenic differentiation of MSCs, along with up-regulating of *Col II* and *aggrecan* expression and Glycosaminoglycan (GAG) production, triggered by these factors: parathyroid hormone-related peptide (PTHrP), *TGF- β* , insulin growth factor (*IGF*)-1 or bone morphogenetic protein (*BMP*)-2 (30–33). Likewise, our previous study demonstrated that the mixture of ASCs and chondrocytes seeded on the scaffolds revealed more excellent capacities of adhesion, migration and proliferation than these on single cell types; meanwhile, these cells co-cultured with *TGF- β* scaffolds displayed extraordinarily curative effects *in vivo* (19). The reports above were all based on the direct contact between chondrocytes and MSCs or ASCs in pellet or scaffolds. Nevertheless, Marie *et al.* found that under the condition of co-culturing ASCs and osteoarthritis (OA) chondrocytes in a minimal medium and using transwell chamber, ASCs had no effect on the proliferation of chondrocytes but extremely decreased camptothecin-induced apoptosis, and chondrocytes in co-culture with ASCs sustained the expression of chondrocytes' markers specific for a mature phenotype

relatively stable, while hypertrophic and fibrotic markers expressions was down-regulated (34). The conclusion could be drawn that the potential growth factors secreted by ASCs after crosstalk with chondrocytes might be accounting for superior cartilage repair and regeneration through the way of paracrine.

Hypoxia was considered into our present study as dominating potential factor to chondrogenic differentiation. No matter in physiological process or pathologic conditions such as tumour progression, hypoxia plays a relatively significant and non-substitutable role. The chondrocytes in body inevitably experience its metabolism and viability under hypoxia. In addition, Gelse *et al.* indicates that chondrogenesis by precursor cells is facilitated in deeper hypoxic zones of cartilage repair tissue and is stimulated by growth factors which enhance *HIF-1 α* activity (35). Moreover, several studies show that hypoxia can enhance the expression of *COL II*, *AGC* and *SOX9* (20,21). Hypoxia has a broader beneficial effect on the chondrocyte phenotype and Lafont *et al.* describes a new pathway to activate the *SOX9* transcription factor independent of *HIF-2 α* , i.e. another new chondrogenesis-modulating protein (36). The above *in vivo* and *in vitro* studies indicate that hypoxia is indispensable for chondrogenesis. Same as chondrocytes, ASCs naturally exist in a low oxygen tension niche, less than 4% O_2 tension in adipose tissue (37,38). Hypoxia is a critical element of this niche, playing a fairly vital role in keeping the characteristics of ASCs. Besides, Jane *et al.* demonstrated that hypoxia (2% O_2) extremely cemented the ASCs chondrogenic differentiation ability as well as increased ASCs stemness marker expression and proliferation rate without altering their morphology and surface markers (25).

In our study, given that an oxygen-limited condition represents the native physiological microenvironment of both ASCs and chondrocytes, results showed that cell

numbers of both ASCs and chondrocytes significantly increased under hypoxia, which may indicate hypoxia promoted the proliferation of ASCs and chondrocytes. Meanwhile, we found that chondrogenic-specific marker genes, i.e. *AGC*, *Col II* and *SOX9*, revealed a slightly higher expression in the low oxygen tension conditions in both ASCs and chondrocytes. Similarly, the proteins expression of *SOX9*, *Col II* and *AGC* up-regulate under hypoxia in co-cultured ASCs and in co-cultured chondrocytes. In addition, the expressions of *Col II* and *AGC* in co-cultured supernatant media under hypoxia were higher than under normoxia both in ASCs and chondrocytes. It confirms that chondrogenic differentiation of ASCs increased under hypoxia in comparison to normoxia. Consistent with our results, numerous previously studies reported that hypoxia could enhance chondrogenic differentiation of ASCs (39,40). It can be concluded that ASCs in co-cultured chondrocytes possess a greater potential to experience chondrogenic differentiation at oxygen levels as low as 2% O₂ than normoxia (21% O₂).

It is well known that hypoxia is closely associated with angiogenesis, through extremely enhancing the expression of angiogenesis-related factors. Therefore, vascular endothelial growth factors (*VEGF*) including *VEGF-A*/*VEGF-B* were investigated in our study. In addition, hypoxia-inducible factor (*HIF-1 α*), induced by low oxygen tension, plays a crucial role in chondrogenesis, cell proliferation and differentiation (41). Previously studies reported that *HIF-1 α* could enhance the expression level of chondrogenic-specific marker genes; conversely, chondrogenic markers expression decreased by the deletion of *HIF-1 α* (42,43). Consistent with our results, *HIF-1 α* was extremely enhanced under hypoxia in comparison to normoxia, at the same time, strengthened in co-culture system compared to mono-culture in both ASCs and chondrocytes. It follows from this that *HIF-1 α* can mediate the expression of chondrogenic marker genes. Both *VEGF-A* and *VEGF-B* in ASCs and chondrocytes were markedly increased under hypoxia condition along with the increasing of *HIF-1 α* , at the same time, compared to mono-culture, they were also highly enhanced after co-culture. Likewise, there has a dramatically increase in the expression of *VEGF* in proliferative chondrocytes *in vitro* under hypoxia, which is demonstrated that is dependent on *HIF-1 α* (44).

In the process of chondrogenesis, there are more growth factors having a strikingly influence on it, mainly including transforming growth factor- β 1 (*TGF- β 1*), bone morphogenetic proteins (*BMP-2/-4/-6*), insulin-like growth factor-1 (*IGF-1*) and fibroblast growth factor (*FGF*). Numerous studies demonstrated that the expression of *Col II*, *aggrecan* and *GAG* in MSCs were increased after co-culture with chondrocytes, occurring

through the factors: *PTHrP*, *TGF- β* , *IGF-1* and *BMP-2* secreted by chondrocytes (30–33). It was demonstrated that a remarkable down-regulation of *TGF- β 1* secretion by chondrocytes and an increase in hepatocyte growth factor (*HGF*) secretion by ASCs potentially involved in the chondroprotective role of ASCs on chondrocytes (34). Our present study showed that *TGF- β 1* decreased in both ASCs and chondrocytes under hypoxia in comparison to normoxia, especially in co-culture. The conclusion can be drawn that hypoxia and co-culture with ASCs might protect chondrocytes. In cartilage extracellular matrix deposition, *BMP-2* might play a significantly crucial role by the crosstalk between *TGF* and *BMP* signalling (45). In comparison to *TGF- β 1* alone, *BMP-4* dramatically augmented the chondrogenic phenotype of ASCs in the differentiation medium (46). Estes *et al.*'s demonstrated that when *BMP-6* together with *TGF- β* markedly enhanced ASCs chondrogenesis through increasing collagen II and *aggrecan* expression almost without up-regulating the expression of collagen type X or other characteristics of a hypertrophic phenotype (47). It is surprising that *BMP-2/-4/-6* in both ASCs and chondrocytes were increased under hypoxia. In addition, *IGF-1* and *FGF-1/-2* were also investigated in our present work, the results showed that all of them have a slightly increase in the low oxygen tension. It is suggested that the over-expression of *IGF-1* combined with dynamic compression could be in favour of ASCs cartilage tissue formation (48). *FGF* is believed to benefit ASCs proliferation while keeping their chondrogenic potential (49). In conclusion, hypoxia and co-culture system can enhance the chondrogenesis of ASCs and protect chondrocytes after crosstalk with chondrocytes.

It is necessary to make reference to some limitations in our study. First, the chondrogenesis specific genes and other related growth factors according to a common gene bank were investigated, but some other growth factors not mentioned might have a notably important influence on chondrogenesis of ASCs after crosstalk with chondrocytes under hypoxia. Secondly, we did not elaborate the precise mechanism about how hypoxia and co-culture system influence both ASCs and chondrocytes. Thirdly, the gene profile of growth factors were detected by semi-quantitative PCR, due to the results could not be fairly accurate, and quantitative polymerase chain reaction (qPCR) should be used in the future.

Acknowledgement

This work was funded by National Natural Science Foundation of China (81470721, 81321002), Sichuan Science and Technology Innovation Team (2014TD0001).

References

- 1 Falchuk KH, Goetzl EJ, Kulka JP (1970) Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. *Am. J. Med.* **49**, 223–231.
- 2 Ferrell WR, Najafipour H (1992) Changes in synovial PO₂ and blood flow in the rabbit knee joint due to stimulation of the posterior articular nerve. *J. Physiol.* **449**, 607–617.
- 3 Kiaer T, Grønlund J, Sørensen KH (1988) Subchondral pO₂, pCO₂, pressure, pH, and lactate in human osteoarthritis of the hip. *Clin. Orthop. Relat. Res.* **229**, 149–155.
- 4 Lund-Olesen K (1970) Oxygen tension in synovial fluids. *Arthritis Rheum.* **13**, 769–776.
- 5 Silver IA (1975) Measurement of pH and ionic composition of pericellular sites. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **271**, 261–272.
- 6 Treuhaft PS, MCCarty DJ (1971) Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. *Arthritis Rheum.* **14**, 475–484.
- 7 Shapiro IM, Tokuoka T, Silverton SF (1991). Energy metabolism in cartilage. In: Hall BK, Newman S, eds. *Cartilage: Molecular Aspects*, pp. 97–130. Boston: CRC Press.
- 8 Wakitani S, Goto T, Young RG, Mansour JM, Goldberg VM, Caplan AI (1998) Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. *Tissue Eng.* **4**, 429–444.
- 9 Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH (2010) Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. *Am. J. Sports Med.* **38**, 1110–1116.
- 10 Ji X, Yang W, Wang T, Mao C, Guo L, Xiao J *et al.* (2013) Coaxially electrospun core/shell structured poly (L-lactide) acid/chitosan nanofibers for potential drug carrier in tissue engineering. *J. Biomed. Nanotechnol.* **9**, 1672–1678.
- 11 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D *et al.* (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315–317.
- 12 Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B *et al.* (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J. Cell. Physiol.* **206**, 229–237.
- 13 Im GI, Shin YW, Lee KB (2005) Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthritis Cartilage* **13**, 845–853.
- 14 Dragoo JL, Samimi B, Zhu M, Hame SL, Thomas BJ, Lieberman JR *et al.* (2003) Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. *J. Bone Joint Surg. Br.* **85**, 740–747.
- 15 Garza-Veloz I, Romero-Diaz VJ, Martinez-Fierro ML, Marino-Martinez IA, Gonzalez-Rodriguez M, Martinez-Rodriguez HG *et al.* (2013) Analyses of chondrogenic induction of adipose mesenchymal stem cells by combined co-stimulation mediated by adenoviral gene transfer. *Arthritis. Res. Ther.* **15**, R80.
- 16 Ahtiainen K, Sippola L, Nurminen M, Mannerström B, Haimi S, Suuronen R *et al.* (2015) Effects of chitosan and bioactive glass modifications of knitted and rolled polylactide-based 96/4 L/D scaffolds on chondrogenic differentiation of adipose stem cells. *J. Tissue Eng. Regen. Med.* **9**, 55–65.
- 17 Waters HA, Geffre CP, Gonzales DA, Grana WA, Szivek JA (2013) Co-culture of adipose derived stem cells and chondrocytes with surface modifying proteins induces enhanced cartilage tissue formation. *J. Invest. Surg.* **26**, 118–126.
- 18 Lee CS, Burnsed OA, Raghuram V, Kalisvaart J, Boyan BD, Schwartz Z (2012) Adipose stem cells can secrete angiogenic factors that inhibit hyaline cartilage regeneration. *Stem Cell Res. Ther.* **3**, 35.
- 19 Li G, Fu N, Xie J, Fu Y, Deng S, Cun X *et al.* (2015) Poly (3-hydroxybutyrate-co-4-hydroxybutyrate) based electrospun 3D scaffolds for delivery of autogeneic chondrocytes and adipose-derived stem cells: evaluation of cartilage defects in rabbit. *J. Biomed. Nanotechnol.* **11**, 105–116.
- 20 Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR (2010) Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell Transplant.* **19**, 29–42.
- 21 Lee HH, Chang CC, Shieh MJ, Wang JP, Chen YT, Young TH *et al.* (2013) Hypoxia enhances chondrogenesis and prevents terminal differentiation through PI3K/Akt/FoxO dependent anti-apoptotic effect. *Sci. Rep.* **3**, 2683.
- 22 He W, Liu X, Kienzle A, Müller WEG, Feng Q (2016) In vitro uptake of silver nanoparticles and their toxicity in human mesenchymal stem cells derived from bone marrow. *J. Nanosci. Nanotechnol.* **16**, 219–228.
- 23 Merceron C, Vinatier C, Portron S, Masson M, Amiaud J, Guigand L *et al.* (2010) Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stem cells. *Am. J. Physiol. Cell Physiol.* **298**, 355–364.
- 24 Portron S, Merceron C, Gauthier O, Lesoeur J, Sourice S, Masson M *et al.* (2013) Effects of in vitro low oxygen tension preconditioning of adipose stromal cells on their in vivo chondrogenic potential: application in cartilage tissue repair. *PLoS ONE* **8**, e62368.
- 25 Choi JR, Pingguan-Murphy B, Wan Abas WA, Noor Azmi MA, Omar SZ, Chua KH *et al.* (2014) Impact of low oxygen tension on stemness, proliferation and differentiation potential of human adipose-derived stem cells. *Biochem. Biophys. Res. Commun.* **448**, 218–224.
- 26 Kanichai M, Ferguson D, Prendergast PJ, Campbell VA (2008) Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1 α . *J. Cell. Physiol.* **216**, 708–715.
- 27 Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.* **5**, 623–628.
- 28 Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D *et al.* (2002) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev.* **111**, 61–73.
- 29 Zhang J, He F, Zhang W, Zhang M, Yang H, Luo ZP (2015) Mechanical force enhanced bony formation in defect implanted with calcium sulphate cement. *Bone Res.* **3**, 14048.
- 30 Acharya C, Adesida A, Zajac P, Mumme M, Riesle J, Martin I *et al.* (2012) Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in co-culture pellets mediate improved cartilage formation. *J. Cell. Physiol.* **227**, 88–97.
- 31 Aung A, Gupta G, Majid G, Varghese S (2011) Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells. *Arthritis Rheum.* **63**, 148–158.
- 32 Fischer J, Dickhut A, Rickert M, Richter W (2010) Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in co-culture during chondrogenesis. *Arthritis Rheum.* **62**, 2696–2706.

- 33 Liu X, Sun H, Yan D, Zhang L, Lv X, Liu T *et al.* (2010) In vivo ectopic chondrogenesis of BMSCs directed by mature chondrocytes. *Biomaterials* **31**, 9406–9414.
- 34 Maumus M, Manferdini C, Toupet K, Peyrafitte JA, Ferreira R, Facchini A *et al.* (2013) Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. *Stem Cell Res.* **11**, 834–844.
- 35 Gelse K, Mühle C, Knaup K, Swoboda B, Wiesener M, Hennig F *et al.* (2008) Chondrogenic differentiation of growth factor-stimulated precursor cells in cartilage repair tissue is associated with increased HIF-1alpha activity. *Osteoarthritis Cartilage* **16**, 1457–1465.
- 36 Lafont JE, Talma S, Hopfgarten C, Murphy CL (2008) Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. *J. Biol. Chem.* **283**, 4778–4486.
- 37 Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE *et al.* (2009) Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* **58**, 718–725.
- 38 Chung HM, Won CH, Sung JH (2009) Responses of adipose-derived stem cells during hypoxia: enhanced skin-regenerative potential. *Expert Opin. Biol. Ther.* **9**, 1499–1508.
- 39 Xu Y, Malladi P, Chiou M, Bekerman E, Giaccia AJ, Longaker MT (2007) In vitro expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. *Tissue Eng.* **13**, 2981–2993.
- 40 Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F (2005) Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. *J. Cell. Physiol.* **204**, 184–191.
- 41 Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS (2001) Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes Dev.* **15**, 2865–2876.
- 42 Amarilio R, Viukov SV, Sharir A, Eshkar-Oren I, Johnson RS, Zelzer E (2007) HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis. *Development* **134**, 3917–3928.
- 43 Malladi P, Xu Y, Chiou M, Giaccia AJ, Longaker MT (2007) Hypoxia inducible factor-1alpha deficiency affects chondrogenesis of adipose-derived adult stromal cells. *Tissue Eng.* **13**, 1159–1171.
- 44 Pfander D, Cramer T, Schipani E, Johnson RS (2003) HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. *J. Cell Sci.* **116**, 1819–1826.
- 45 Shen J, Li S, Chen D (2014) TGF- β signaling and the development of osteoarthritis. *Bone Res.* **2**, 14002.
- 46 Gong T, Xie J, Liao J, Zhang T, Lin S, Lin Y (2015) Nanomaterials and regenerative medicine. *Bone Res.* **3**, 15029.
- 47 Estes BT, Wu AW, Guilak F (2006) Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis Rheum.* **54**, 1222–1232.
- 48 Li J, Zhao Q, Wang E, Zhang C, Wang G, Yuan Q (2012) Dynamic compression of rabbit adipose-derived stem cells transfected with insulin-like growth factor 1 in chitosan/gelatin scaffolds induces chondrogenesis and matrix biosynthesis. *J. Cell. Physiol.* **227**, 2003–2012.
- 49 Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A *et al.* (2007) Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J. Cell. Physiol.* **212**, 702–709.