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# In vitro osteogenesis of human adipose-derived stem cells by coculture with human umbilical vein endothelial cells

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ABSTRACT

Adipose-derived stem cells (ASCs) have been successfully applied in treating bone defects both in animals and humans and promoted osteogenesis in vivo significantly. However, the mechanism of in vivo osteogenesis of ASCs was still little known, we hypothesized that this was mediated in part by interaction between implanted ASCs and local vein endothelial cells. In this study, human adipose-derived stem cells (hASCs) and human umbilical vein endothelial cells (HUVEC) were isolated and characterized. Cells were then either cultured alone or cocultured. Alkaline phosphatase (ALP) staining, quantitative measurement of ALP activity and Alizarin staining of hASCs cultured alone, HUVEC cultured alone and cells cocultured demonstrated that osteogenic differentiation of cocultured cells increased obviously. Osteocalcin (OC) expression of hASCs cocultured with HUVEC showed an obvious raise than hASCs cultured alone. HUVEC cultured alone showed BMP-2 secretion and increased with culturing time. Real-time PCR of the cocultured cells showed four osteogenic differentiation related genes raised with culturing time, while two adipogenic differentiation related genes showed a slightly decrease with culturing time. Results of our study with different culture models showed that in vitro osteogenesis of hASCs was enhanced by coculture with HUVEC which secreted BMP-2. This study not only provided us with an in vitro model of studying interaction between cells, but also helped us to understand the in vivo therapeutic mechanisms of ASCs.



## 1. Introduction

Skeletal defects resulting from tumor resection, trauma, pathological degeneration or congenital deficiency is an important issue for orthopedic surgery. The incidence of skeletal deficits grows as the average age of population increases. The gold standard treatment for skeletal repair involves the use of autogenous bone grafts [1]. However, this contains shortcomings such as donor site damage and limited tissue resources. Allograft is also used to repair skeletal defects for their osteogenicity but with limitations such as disease transfer risk and immunological rejection.

Progress in stem cell research has led to the utilization of stem cells in bone tissue regeneration. Much research has focused on mesenchymal stem cells isolated from bone marrow. Mesenchymal stem cells are adult stem cells that can be isolated from patients themselves thus could refrain from immunological reaction and without ethical problems either [2]. But bone marrow aspiration is especially painful for patients and yields low amount of mesenchymal stem cells.

More recently, adipose-derived stem cells (ASCs), a type of mesenchymal stem cells isolated from adipose tissue have received great attention. They can be easily harvested by liposuction, are available in large numbers, show a strong multi-differentiation ability, attach and proliferate rapidly in culture, making them an idea cell source in orthopedic related research [3]. Moreover, ASCs secrete potent growth factors such as fibroblast growth factor-2 as well as vascular endothelial growth factor (VEGF), showing properties of stimulating angiogenesis which is of vital importance for osteogenesis [4].

Numerous studies have been performed to identify the therapeutic effect of ASCs applied in bone defects in animals [5–7]. For example, Levi and his coworkers [8] demonstrated in their research that undifferentiated hASCs presented effective bone regeneration ability by treating critical-sized (4 mm) non-healing mouse calvarial defects. In addition, one case report showed that transplantation of ASCs promoted osteogenesis in human significantly [9]. Mesimaki and coworkers [10] showed in their study that

*Abbreviations:* ASCs, adipose-derived stem cells; hASCs, human adipose-derived stem cells; HUVEC, human umbilical vein endothelial cells; ALP, alkaline phosphatase; OC, osteocalcin; BMP-2, bone morphogenic protein-2; PPARγ, Peroxisome proliferator-activated receptor γ; AP2, fatty acid binding protein.

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ectopic bone was successfully produced using autogenous ASCs in microvascular reconstruction surgery.

However, little is known about the in vivo osteogenic mechanism of ASCs in context of bone formation, we hypothesized that in vivo osteogenesis of ASCs was mediated in part by local interaction with venous endothelial cells. To elucidate interactions between hASCs and human endothelial cells, we designed an experiment in which human umbilical endothelial cells (HUVEC) and hASCs were cultured in different models. In this study, hASCs and HUVEC were relatively isolated, characterized, cultured alone or cocultured. Alkaline phosphatase (ALP) staining and Alizarin staining of hASCs cultured alone group, HUVEC cultured alone group, and cocultured group demonstrated obvious in vitro osteogenesis of the cocultured group in comparison with the other two groups. Quantitative measurement of ALP activity of these three groups also supported the staining results. Standardized osteocalcin (OC) expression levels of hASCs cultured alone group and hASCs cocultured with HUVEC group showed a statistical difference 7 days after cell culture and showed a greater difference 12 days after cell culture which testified in vitro osteogenesis of hASCs when cocultured with HUVEC. Bone morphogenic protein-2 (BMP-2) expression of HUVEC cultured alone group was measured and increased with culture time. Real-time PCR of the cocultured cells showed four osteogenic related genes increased with culture time and two adipogenic related genes declined slightly with time, which also testified previous results. In summary, this study demonstrated that in vitro osteogenesis of hASCs was enhanced by coculture with HUVEC which secreted BMP-2. This study suggested that the in vivo therapeutic function of ASCs was mediated at least in part by local interaction with endothelial cells.

## 2. Materials and methods

### 2.1. Isolation, characterization, and multi-differentiation ability test of hASCs

Human subcutaneous adipose tissue was obtained from four female patients (31–44 years old) who undergoing surgery following their informed consent as described by Bunnell et al. [11]. All the procedures were approved by the ethical commitment of Wuhan Union Hospital. Cell culture media was replaced every third day. On reaching complete confluence, cells were plated at 1:2 dilutions under the same culture condition. Cells passaged 4–7 times were used in the experiments.

HASCs passaged 4 times were resuspended in PBS and stained using antibodies HLA-DR-FITC, CD45-PERCP, CD29-PE and CD44-APC for 20 min, isotype-matched normal IgG were used as controls. After staining, the cells were washed twice in PBS containing 2% fetal bovine serum and analyzed using a standard Becton–Dickinson FACSAria instrument (BD, San Jose, CA, USA). All antibodies and isotype controls were purchased from BD Sciences.

For osteogenic differentiation, hASCs were cultured in the presence of DMEM/F-12 supplemented with 10% FBS, 0.1 mM dexamethasone (Sigma), 10 mM β-glycerolphosphate (Sigma) and 50 mM ascorbic acid (Sigma) for about 2 weeks. At the end of incubation, osteogenic differentiation was assayed by Alizarin red staining. For adipogenic differentiation, hASCs were cultured in the presence of DMEM supplemented with 10% FBS, 1 mM dexamethasone (Sigma), 0.5 mM methyl-isobutyl-xanthine (Sigma), 10 mg/ml insulin (Invitrogen, Carlsbad, CA) and 100 mM indomethacin (Sigma) for 3 weeks. At the end of the incubation, adipogenic differentiation was assayed by Oil-Red-O staining.

### 2.2. Isolation and characterization of HUVEC

Umbilical cords were obtained from three healthy delivery women under their informed consent, all the procedures were approved by the ethical commitment of Wuhan Union Hospital. The endothelial cells were extracted from human umbilical veins as described by Bruno et al. [12]. The culture media was replaced every other day and cells passaged 0–3 times were used in all experiments.

HUVEC was characterized by increased metabolism of Ac-LDL (acetylated low density lipoprotein). Ac-LDL labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was utilized to stain the cellular cytoplasm and identify the cells. DAPI (4′,6-diamidino-2-phenylindole) was utilized to stain the cellular nucleus.

### 2.3. Coculture of hASCs and HUVEC

When cells reached 80–90% confluence, hASCs and HUVEC were trpsinized separately and subcultured in 24-well plate at a ratio of 1:1 during the same seeding phase. HASCs and HUVEC cultured alone in the same culture medium served as control groups. The medium was changed every other day.

### 2.4. ALP staining and Alizarin staining

ALP was selected as an early stage osteogenic differentiation marker. HASCs cultured alone group, HUVEC cultured alone group, and cells of the cocultured group were relatively stained 7 days after cell culture as described previously [13].

After 14 days of culture, the three groups were separately stained by the Alizarin red to assess mineralization. First cells were fixed in 70% ice-cold ethanol for 1 h, then rinsed with double distilled water and stained with 40 mM Alizarin red at pH 4.2 for 10 min with gentle agitation. Finally the stained cells were washed with double distilled water repeatedly and rinsed for 15 min with 1 × PBS with gentle agitation.

### 2.5. Quantitative measurement of ALP activity

Quantitative measurement of ALP activity was determined 4 and 7 days after cell culture by measuring the relative amount of *p*-nitrophenol phosphate substrate. Cell lysates were mixed with alkaline buffer solution and gently shaken for 10 min. ALP substrate was added at room temperature for 30 min, and the reaction was subsequently stopped with addition of 0.05 N NaOH, absorbance at 405 nm was recorded and was used to represent the ALP activity, OD value at a wavelength of 405 nm was normalized by the cellular protein concentration. The quantitative measurement of cellular proteins was done following the method of Lowry et al. [14].

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

After 7 days and 12 days of culture, OC expressions of hASCs cultured alone group and hASCs cocultured with HUVEC group were relatively measured by ELISA with a Mid-Tact OC ELISA kit (Biomedical Technologies). 24 h prior to measuring OC levels at each time point, the medium was replaced with fresh culture medium. After ELISA measurements, hASCs were isolated from the cocultured cells by immunomagnetic separation. Cells were digested by trypsin (0.05%), pelleted and then resuspended in PBS. Fifteen micrograms anti-human CD31 cocktail (Tianjin Biochip Corporation) was added to the cocultured cells and maintained for 10 min. Magnetic nanoparticles were next added to the cells and incubated for a total of 10 min. The magnet was used to isolate hASCs from the cocultured cells. HASCs were isolated and measured for total protein level as described above. OC concentration divided by protein level was denoted as the standardized OC level.

**Table 1**
RT-PCR primers used in the experiments.

| Gene | Primer | Product size | Accession No. |
|---|---|---|---|
| Runx2 | F: CCAGATGGGACTGTGGTTACTG<br>R: TTCCGGAGCTCAGCAGAATAA | 76 | NM_001024630F |
| Osteopontin | F: TGAGCATTCCGATGTGATTGA<br>R: TGTGGAATTCACGGCTGACTT | 71 | NM_00104058F |
| Osteocalcin | F: TGTGAGCTCAATCCGGACTGT<br>R: CCGATAGGCCTCCTGAAAGC | 63 | NM_199173F |
| BMP2 | F: GCCCTTTTCCTCTGGCTGAT<br>R: TTGACCAACGTCTGAACAATGG | 140 | NM_001200F |
| PPARγ | F: TTCAGAAATGCCTTGCAGTG<br>R: CCAACAGCTTCTCCTTCTCG | 84 | NM_138712 |
| AP2 | F: TACTGGGCCAGGAATTTGAC<br>R: GGACACCCCCATCTAAGGTT | 78 | J02874 |
| RPL13a | F: CATAGGAAGCTGGGAGCAAG<br>R: GCCCTCCAATCAGTCTTCTG | 75 | NM_01242 |

Bone morphogenetic protein-2 (BMP-2) expression of HUVEC cultured alone group was relatively measured after 2, 4, 6, 8, and 10 days of cell culture with a human BMP-2 ELISA Development Kit (PEPROTECH). Twenty four hours prior to measuring BMP-2 levels at each time point, the medium was replaced with fresh culture medium.

### 2.7. Real-time quantitative PCR of the cocultured group

After 5 and 10 days of coculture, Total RNA was isolated with Trizol (Invitrogen) and used to synthesize cDNA with the superscript II cDNA synthesis kit (Invitrogen). The osteogenic differentiation markers and adipogenic differentiation markers were assessed by quantitative Real-time PCR using SYBR green master mix (ABI). Ribosomal protein L13a (RPL13a) was amplified as an internal control. The primer information was seen in Table 1. Real-time PCR were run in triplicate on 384-well plates and gene expression was analyzed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

### 2.8. Statistical analysis

All experiments were done three times independently. Data are expressed as mean and SD. The significance of difference was determined using Student *t*-test. Differences with $p$ values <0.05 were considered statistically significant. All analysis were performed with SPSS 13.0.

## 3. Results

### 3.1. Flow cytometry, morphology and multi-differentiation ability test of hASCs

To characterize the cell-surface antigen profile of hASCs, the expression of surface markers were analyzed by flow cytometry at passage four. Results of the flow analysis showed that cells positively expressed CD29 and CD44, and negatively expressed HLA-DR, CD45 (Fig. 1). In the first passage of culture, the cells proliferated slowly. By passage three, a homogeneous population of flat and fibroblast-like cells was obtained (Fig. 2A1). In order to determine the multipotency of hASCs, hASCs used in this study were cultured in either osteogenic or adipogenic differentiation medium. Osteogenic potential of hASCs cultured in osteogenic medium for two weeks was confirmed by the colonies positive for Alizarin red staining (Fig. 2A2).Culture of hASCs in adipogenic medium for three weeks resulted in the development of several clusters of adipocytes containing intracellular lipid vacuoles, which stained positive with Oil Red (Fig. 2A3).

### 3.2. Characterization of HUVEC

Cell nucleus of HUVEC was stained by DAPI (4′,6-diamidino-2-phenylindole) and showed a blue color(Fig. 2B1)under the fluorescence microscope after being excited by fluorescence at a wavelength of 380 nm. At the same time, cytoplasm of HUVEC was stained by the uptake of DiI-Ac-LDL (fluorescent acetylated low-density lipoprotein) by the "scavenger cell pathway" of LDL according to the operative conditions of Voyta et al. [15]. The cytoplasm of HUVEC showed a red color (Fig. 2B2) under the fluorescence microscope after being excited by fluorescence at a wavelength of 514 nm. Almost all of the HUVEC were positive stained by both DAPI and DiI-Ac-LDL (Fig. 2B3).

### 3.3. ALP staining and Alizarin staining

All the ALP staining was done 1 week after cell culture. HUVEC cultured alone showed a light blue color after ALP staining (Fig. 2C1), hASCs showed a little deeper blue color after ALP staining (Fig. 2C2), while cells of the coculture group showed the deepest blue color after ALP staining (Fig. 2C3).

Calcium deposition of each group was measured by Alizarin red staining. Cells of each group were cultured for 2 weeks before staining. Cells were hydrated to 70% alcohol and rinsed rapidly in distilled water, then stained by alizarin red for 8 min. Finally cells were washed with distilled water five times before rinsed with 1× PBS solution. Calcium deposition of HUVEC cultured alone group (Fig. 2D1) and hASCs cultured alone group (Fig. 2D2) was not detected under microscope by Alizarin red staining. While small plaques of calcified extracellular matrix of the cocultured group can be obviously detected under microscope (Fig. 2D3 100× and Fig. 2D4 200×).

### 3.4. Quantitative measurement of ALP activity

Quantitative measurement of ALP activity was done 4 days and 7 days after cell culture. ALP activity, represented by the OD value at a wavelength of 405 nm, was divided by total cellular protein content to denote the normalized ALP activity that can be compared between groups. Each group showed a different level of ALP activity and ALP activity of each group increased during cell culture, HUVEC showed a higher ALP activity than that of hASCs 4 days after cell culture, but showed a lower ALP activity than that of hASCs seven days after cell culture. There was a significant increase of normalized ALP activity in the cocultured group than that of the cultured alone groups both 4 days and 7 days after cell culture (Fig. 3A).

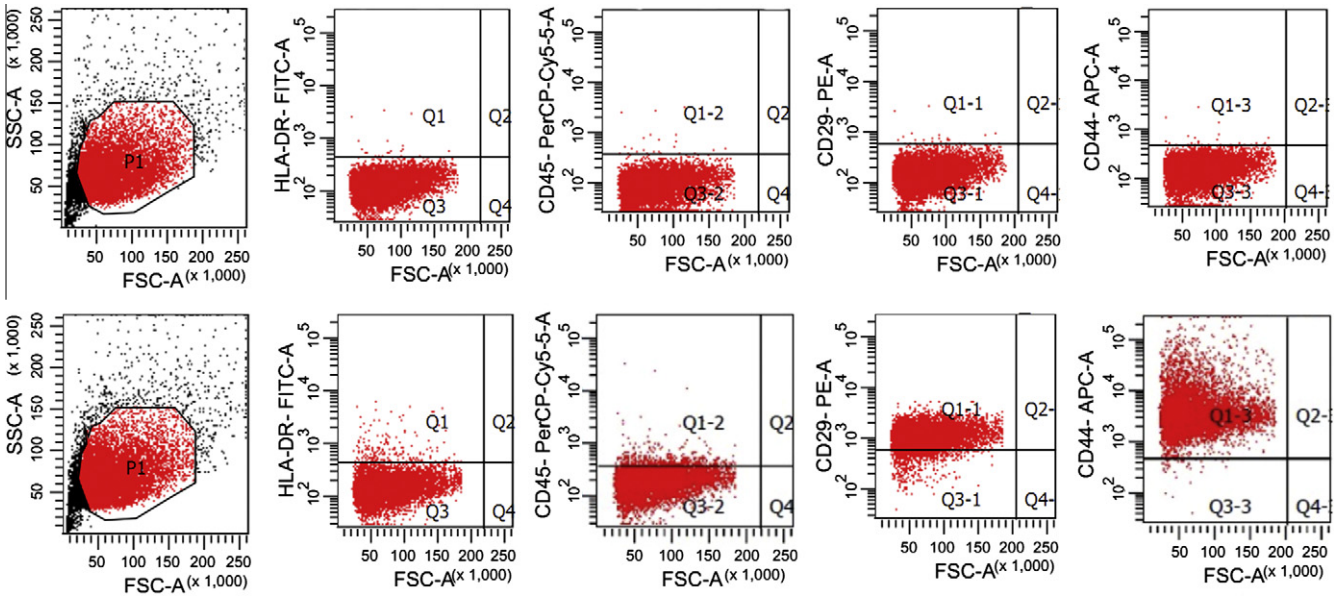


**Fig. 1.** Immunophenotype of hASCs. HASCs were stained using corresponding antibodies, isotype-matched normal IgG were used as controls. Results showed that isolated hASCs positively expressed CD29 and CD44, and negatively expressed HLA-DR, CD45.

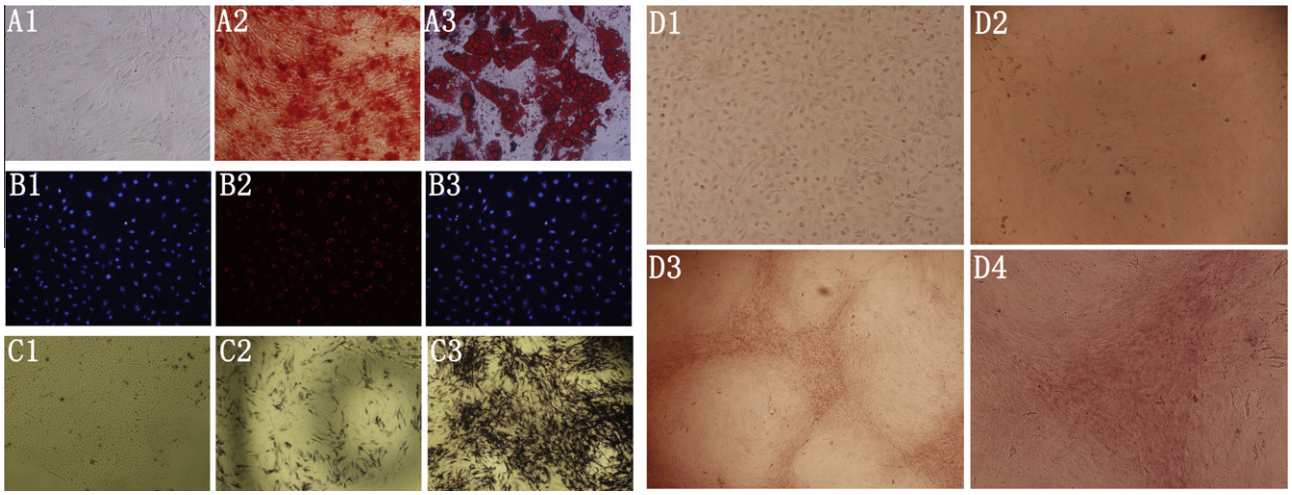


**Fig. 2.** Staining of cells cultured alone or cocultured. Cultured hASCs showed flat and fibroblast-like cells by passage 3 (A1). Osteogenic differentiatiated hASCs were positively stained by Alzarin red (A2). Adipogenic differentiatiated hASCs were positively stained by Oil Red (A3). Cell nucleus of HUVEC was positively stained by DAPI and showed a light blue color (B1). Cytoplasm of HUVEC was positively stained by DiI-Ac-LDL and showed a red color (B2). Merged image showed that most isolated cells were positively by both DAPI and DiI-Ac-LDL (B3). HUVEC cultured alone showed a light blue color after ALP staining (C1). HASCs showed a little deeper blue color after ALP staining (C2). The cocultured cells showed the deepest blue color after ALP staining (C3). Calcium deposition of HUVEC cultured alone group (D1) and hASCs cultured alone group (D2) was not detected after Alizarin red staining. Small plaques of calcified extracellular matrix of the cocultured cells could be obviously detected under microscope (D3 × 100 and D4 × 200). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.5. OC and BMP-2 expression analysis with ELISA

Seven days and 12 days after cell culture, OC production of the HUVEC group were measured, but no detectable levels were present (data not shown). Seven days and 12 days after cell culture, production of OC secreted by hASCs cocultured with HUVEC was also measured and was higher than that of hASCs cultured alone with statistical significance. OC was normalized by detected OC concentration divided by isolated hASCs protein level (Fig. 3B).

Two days, 4 days, 6 days, 8 days and 10 days of HUVEC culture, BMP-2 production was relatively measured with ELISA. Results showed an increased production of BMP-2 with time (Fig. 3C).

### 3.6. Real-time quantitative PCR of the co-cultured group

Cbaf-1/Runx2, osteopontin, osteocalcin and BMP-2 were chosen as osteogenic differentiation related genes, while PPARγ and AP2 were chosen as adipogenic differentiation related genes. Gene expressions of the cocultured cells were relatively measured with Real-time PCR 5 days and 10 days after culture. Results showed that four osteogenic differentiation related genes increased with time with statistical difference (Fig. 4A–D) while two adipogenic related genes showed a slightly decrease with time (Fig. 4E–F). Ribosomal protein L13a (RPL13a) was amplified as an internal control.

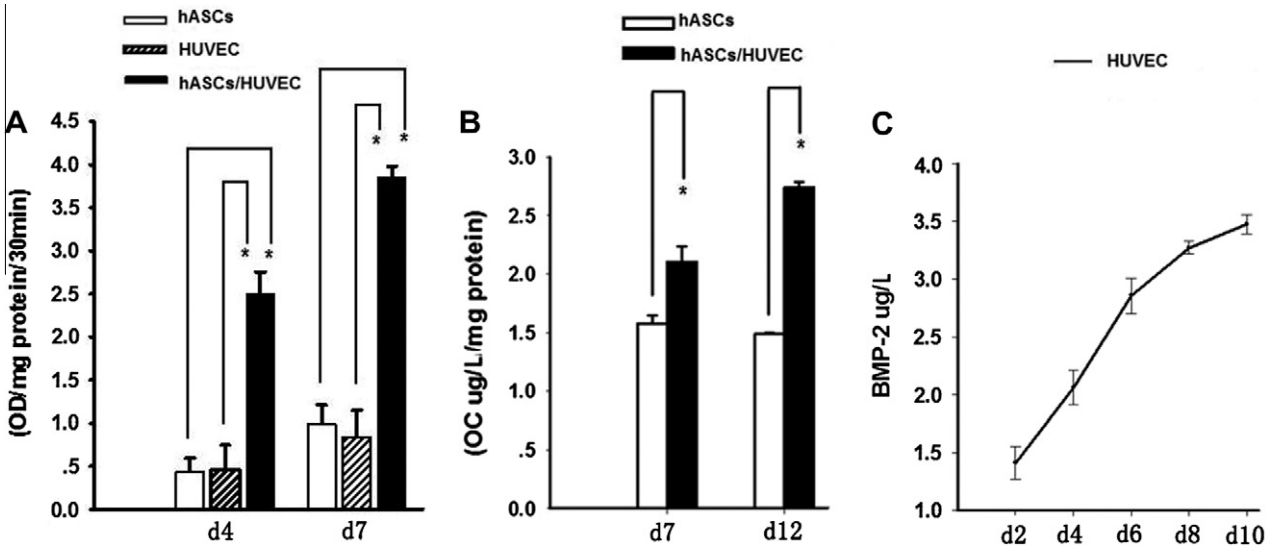


**Fig. 3.** Quantitative ALP measurement and ELISA measurement. Normalized ALP activity in the cocultured group (hASCs/HUVEC) was greater than that of the two cultured alone groups (hASCs and HUVEC) both 4 days and 7 days after cell culture with statistical difference (A). Normalized OC levels of cocultured cells (hASCs/HUVEC) were greater than the hASCs cultured alone group both 7 days and 12 days after cell culture with statistical difference (B). HUVEC cultured alone group showed secretion of BMP-2 and BMP-2 levels increased with culture time (C). (∗ denoted that $p < 0.05$).

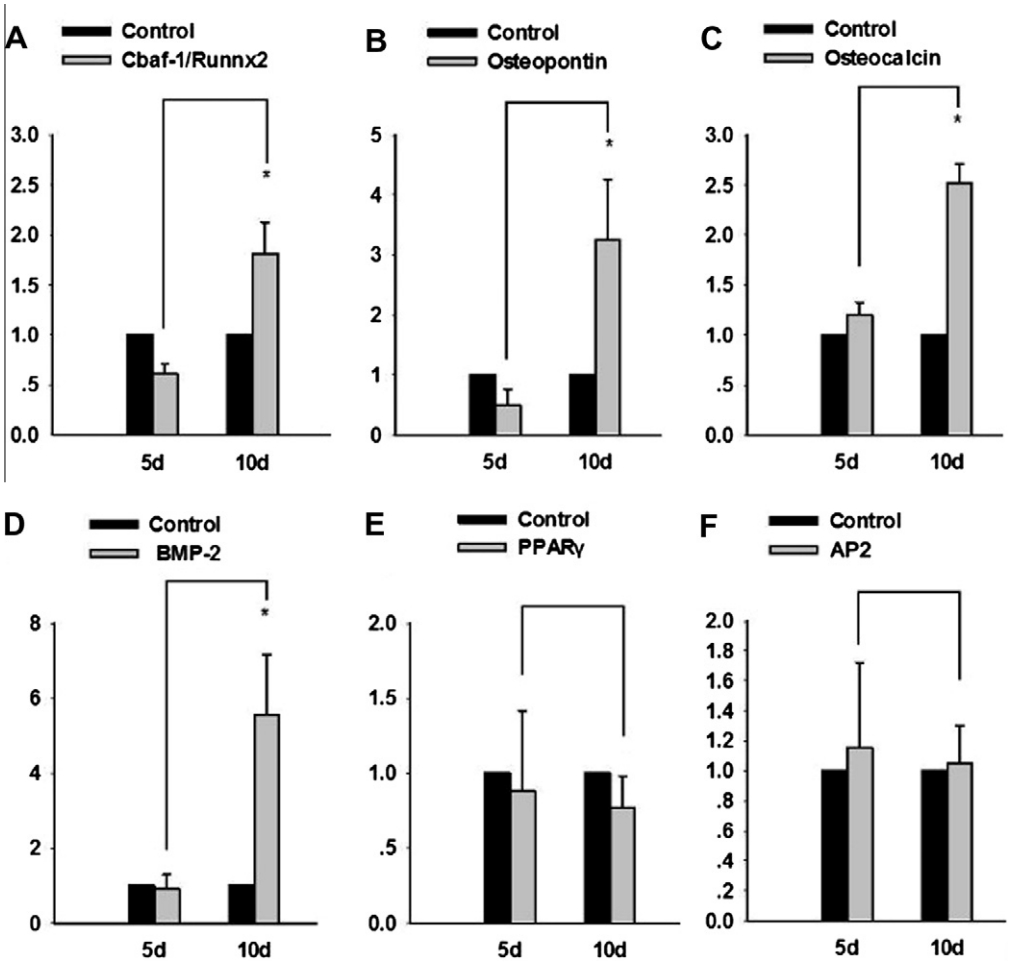


**Fig. 4.** RT-PCR results. Four osteogenic differentiation related markers Cbaf-1/Runx2 (A), osteopontin (B), osteocalcin (C) and BMP-2 (D) showed an increased expression 10 days after culture than that 5 days after culture with statistical difference. While two adipogenic differentiation related genes PPARγ and AP2 showed a decreased expressions 10 days after culture than that 5 days after culture. (∗ denoted that $p < 0.05$).

## 4. Discussion

ASCs have been successfully applied in treating bone defect both in animals and humans, we postulated that interaction between transplanted ASCs and local vein endothelial cells is an integral contributor of the therapeutic effect of ASCs. This study tried to elucidate the interaction between hASCs and HUVEC using in vitro models. HASCs and HUVEC were relatively isolated and identified. HASCs and HUVEC were then cultured alone or cocultured. Results of our experiment showed that in vitro osteogenesis of hASCs was enhanced by coculture with HUVEC.

In this study, hASCs were isolated, cultured and sub-cultured. By passage three, a homogeneous population of flat and fibroblast-like cells was obtained. The surface marker expressions were analyzed by flow cytometry and flow results showed that these cells positively expressed CD29 and CD44, and negatively expressed HLA-DR, CD45, which were consistent with previous studies [16,17]. In order to further characterize hASCs, the multi-differentiation ability was also tested, cells were cultured in either osteogenic or adipogenic differentiation medium [18]. Results showed that hASCs isolated and cultured in this study have the capacity of osteogenic differentiation and adipogenic differentiation.

HUVEC were isolated, cultured and characterized by the uptake of DiI-Ac-LDL (fluorescent acetylated low-density lipoprotein) [19]. Results showed that almost all HUVEC were stained positively by both DAPI and DiI-Ac-LDL, this result showed that HUVEC were perfectly isolated and cultured.

ALP is a membrane-bound enzyme abundant early in osteogenesis of human marrow stromal cells, and increased ALP levels were correlated with enhanced osteogenesis [20]. In this study, ALP staining was done 1 week after cell cultures. Results of ALP staining showed that HUVEC and hASCs expressed low levels of ALP, but cocultured cells showed a substantial increase in ALP expression which demonstrated an early phase of osteogenic differentiation. Alizarin staining was done 2 weeks after cell cultures. HUVEC cultured alone group and hASCs cultured alone group showed no existence of calcium deposition, while cells of the cocultured group showed relatively obvious calcium deposition which testified bone mineralization.

Quantitative measurements of ALP activity were done 4 days and 7 days after cell cultures. Results showed that ALP activity of HUVEC was higher than that of hASCs 4 days after cell culture, but was lower than that of hASCs 7 days after cell culture. Cocultured group both 4 days and 7 days after cell culture showed an obvious increase in ALP activity compared with the HUVEC and hASCs cultured alone groups with significant difference. ALP activities of these three groups 7 days after cell culture were higher than those 4 days after cell culture. The quantitative measurement of ALP activity further testified results of the ALP staining.

OC is a late bone marker that is secreted only by osteoblasts and represents differentiation toward osteoblastic lineage and extracellular mineral matrix maturation [21]. HASCs cultured alone and hASCs cocultured with HUVEC were compared for OC expression. Seven days and 12 days after cell culture, OC expression of these two groups both showed significant difference and OC expression increased 12 days after cell culture than 7 days. This demonstrated that hASCs cultured in the presence of HUVEC were at a more mature state along the osteogenic differentiation pathway.

BMPs are members of the TGF-β superfamily and initiate their signaling cascade through types I and II BMP receptors and subsequently form a signaling cascade such as phosphorylation of smad1, 5 and 8 [22]. Rosen described that BMP-2 is responsible for the direct differentiation of mesenchymal stem cells into osteoblastic lineage which accelerates and improves the bone regeneration [23]. BMP-2 expression of HUVEC cultured alone group was relatively measured 2 days, 4 days, 6 days, 8 days and 10 days after cell culture. Results showed that HUVEC cultured alone group showed a rapid increase in BMP-2 secretion with culture time. We thought that in vitro osteogenesis of hASCs was promoted by BMP-2 secreted by HUVEC as one possible mechanism.

Finally, four osteogenic related differentiation genes and two adipogenic related differentiation genes of the cocultured cells were assayed by Real-time PCR. Cbaf-1/Runnx2, a master gene for osteogenic differentiation, showed a significant increase 10 days after cell culture than that expressed 5 days after cell culture. Osteocalcin, a late osteogenic differentiation marker which is secreted by osteoblastic lineage cells, osteopontin (OPN), an important component of the mineralized extracellular matrices of skeletal tissues, both raised 10 days after cell culture than 5 days after cell culture. BMP-2 also revealed an increase with time. While adipogenic related genes AP2 and PPARγ showed slightly decrease with time. The Real-time PCR demonstrated that coculture of hASCs and HUVEC preferentially promoted selectively in vitro osteogenic differentiation of hASCs but not adipogenic differentiation.

In summary, this study provided us with an in vitro model of studying interaction between cells and also revealed that in vitro osteogenesis of hASCs was enhanced by cocultured with HUVEC. This study pointed out that the in vivo therapeutic effect of ASCs was mediated at least in part by interaction between local venous endothelial cells and implanted ASCs. However, more strictly designed in vivo studies are needed to further elucidate the in vivo therapeutic mechanism of ASCs.

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