

RESEARCH ARTICLE

Human amnion-derived mesenchymal stem cells promote osteogenic and angiogenic differentiation of human adipose-derived stem cells

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Abstract

Tissue engineering using suitable mesenchymal stem cells (MSCs) shows great potential to regenerate bone defects. Our previous studies have indicated that human amnion-derived mesenchymal stem cells (HAMSCs) could promote the osteogenic differentiation of human bone marrow mesenchymal stem cells (HBMSCs). Human adipose-derived stem cells (HASCs), obtained from adipose tissue in abundance, are capable of multi-lineage differentiation. In this study, the effects of HAMSCs on osteogenic and angiogenic differentiation of HASCs were systematically investigated. Proliferation levels were measured by flow cytometry. Osteoblastic differentiation and mineralization were investigated using chromogenic alkaline phosphatase activity (ALP) activity substrate assays, Alizarin red S staining, real-time polymerase chain reaction (real-time PCR) analysis of osteogenic marker expression, and Western blotting. We found that HAMSCs increased the proliferation and osteoblastic differentiation of HASCs. Moreover, enzyme-linked immunosorbent assay (ELISA) and human umbilical vein endothelial cells (HUVECs) tube formation suggested HAMSCs enhanced angiogenic potential of HASCs via secretion of increased vascular endothelial growth factor (VEGF). Thus, we conclude that HAMSC might be a valuable therapeutic approach to promote HASCs-involved bone regeneration.

Introduction

Effective reconstruction of bone defects resulting from trauma and surgical resection is becoming a major clinical challenge in maxillofacial surgery [1–3]. With the development of regenerative medicine, tissue engineering utilizing optimal scaffolds seeded with mesenchymal stem cells has shown a promising potential in generating new bone tissue [4, 5]. Human adipose-derived stem cells (HASCs) can be obtained from adipose, a highly abundant tissue and easily accessible pool of stem cells [6–8]. HASCs are capable of self-renewal and differentiation

into several distinct cell lineages[9]. HASCs are known to present good bone regenerative capacity [10]. Besides, the pericyte-like phenotype formed by HASCs has been shown to play a role in blood vessels maturation and remodeling as an autologous cell source [11–13]. By far, gene therapy, cytokines and Chinese herb extracts have been used to enhance the osteogenic and angiogenic differentiation of HASCs[14–16]. However, disadvantages such as individual differences and easy degradation limit their application.

Human amnion-derived mesenchymal stem cells (HAMSCs) can be obtained from human term placenta, a highly abundant tissue and valuable source of stem/progenitor cells[17]. HAMSCs have potential of differentiation into several cell types including bone, cartilage, fat, and muscle[18]. They are associated with low anti-inflammatory properties and fewer ethical issues than other sources of stem cells, thus providing considerable benefits in bone tissue engineering [19]. Our previous study found that HAMSCs were capable of providing a conducive environment to drive osteogenic differentiation of human bone marrow mesenchymal stem cells (HBMSCs)[20]. Moreover, HAMSCs are able to augment blood perfusion and increase intraneural vascularity[21], thus possessing higher angiogenic property compared to HASCs[22]. Considering the excellent osteogenic and angiogenic ability of HAMSCs, we hypothesize that an appropriate cell-cell culture system may effectively promote both osteogenesis and angiogenesis in HASCs. In the present study, we used a transwell coculture system to evaluate the *in vitro* effects of HAMSCs on the proliferation, osteogenic and angiogenic differentiation of HASCs. Moreover, the role of potential signal pathways involved in this process was also investigated.

Materials and methods

Chemicals and reagents

Trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco[®] Life Technologies. The Alizarin red S (pH 4.4), protein assay kit, lysis buffer, ALP and bicinchoninic acid (BCA) assay kits were purchased from the Jiancheng Corp (Nanjing, China). EBM (endothelial basal medium) was purchased from ScienCell[®] (San Diego, USA). Penicillin G-streptomycin sulfate, α -minimum essential medium (α MEM), dexamethasone, β -glycerophosphate ascorbic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Six-well culture plates and transwells (6-Well Millicell Hanging Cell Culture Inserts, 0.4 μ m, PET) were purchased from Millipore[®] (Bedford, MA, USA). Human VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Growth factor-reduced Matrigel was purchased from BD Bioscience (San Diego, CA). EGM-2 BulletKit was purchased from Lonza (Walkersville, MD, USA). The mouse anti-rabbit IgG (L27A9) mAb, anti-mouse IgG (HRP-linked Antibody #7076), phospho-p44/42 (p-ERK1/2) MAPK rabbit mAb, p44/42 MAPK (ERK1/2) rabbit mAb, phospho-p38 (p-p38) MAPK (Thr180/Tyr182) (D3F9) rabbit mAb, p38 MAPK (D13E1) rabbit mAb, RUNX2 (D1L7F) rabbit mAb, phospho-SAPK/JNK (p-JNK) (Thr183/Tyr185) (81E11) Rabbit mAb, SAPK/JNK (JNK) antibody (#9252), and β -Actin (13E5) Rabbit mAb were purchased from Cell Signalling Technology. The anti-Osteocalcin (OCN) antibody (ab133612), anti-Collagen I (COL1) antibody (ab6308), anti-VEGF Receptor 1 (VEGFR1) antibody (ab32152), and anti-Angiogenin (ab10600) were purchased from Abcam. Other reagents used were of the highest commercial grade available.

Cell culture

HASCs were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HUVECs were purchased from the China Infrastructure of Cell Line Resources (Beijing,

China). Isolation of HAMSCs was performed following the pancreatin/collagenase digestion method[23–25]. HASCs and HAMSCs were cultured in 60-mm plates in α MEM supplemented with 100 U/L penicillin, 100 mg/L streptomycin, and 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. HUVECs were cultured in 60-mm plates in EBM containing 1% FBS and EGM-2 BulletKit in a humidified atmosphere of 5% CO₂ at 37°C. Cells from passage 3 were used and culture medium was changed every 3 days. The study protocols were approved by the Ethics Committee of the School of Stomatology, Nanjing Medical University, China (NO. PJ2013-037-001). Informed consent was obtained from all the participants enrolled in this study.

The coculture system

A transwell coculture system was used to investigate the effects of HAMSCs on HASCs as described previously[26]. HASCs were seeded at an initial cell density of 5×10^4 cells/cm² in 6-well culture plates. Transwells were placed in other 6-well culture plates and HAMSCs were seeded at increasing HASCs: HAMSCs ratios (5×10^4 cells/transwell, 10×10^4 cells/transwell and 15×10^4 cells/transwell). HASCs were subjected to a 24 h treatment with serum-free medium following the attachment of the cells (approximately 12 h). After washing with PBS, transwells containing HAMSCs were transferred into the corresponding wells of the 6-well culture plate containing HASCs to create the HASCs/HAMSCs transwell coculture system. HASCs in wells with transwells served as the treatment groups, while HASCs without transwells were used as the control groups.

Flow cytometry

The effects of HAMSCs on HASCs proliferation was measured by flow cytometry at 1, 3 and 5 days. Briefly, after starvation in serum-free medium for 24 h, HASCs were washed with PBS. Transwells containing HAMSCs were moved into the corresponding wells of the 6-well culture plate containing HASCs, and the medium was replaced with culture medium containing 10% FBS. HASCs were harvested at day 1, 3 and 5 and fixed with 75% ice-cold ethanol at 4°C for 30 min in the dark. DNA content was measured by a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the cell cycle fractions (G₀, G₁, S, and G₂ M phases) were processed using CellQuest Pro software (BD Biosciences). Data was analyzed by ModFitLT 3.2 (verity software house, USA).

ALP activity and mineralized matrix formation

After transwells containing HAMSCs were moved into the corresponding wells of the 6-well culture plate containing HASCs, both the cell types were cultured in osteogenic medium (OS) containing 10 mM β -glycerophosphate, 100 nM ascorbic acid, and 100 nM dexamethasone. HAMSCs, HASCs, and HASCs/HAMSCs groups were subjected to ALP activity assays and Alizarin red staining. ALP activity assay was performed using an ALP assay kit according to the manufacturer's protocols at 7 and 14 days. Alizarin red staining was performed at day 21 using 40 mM Alizarin red S (pH 4.4) for 10 min at room temperature. Following rinsing with PBS, mineralized nodules were visualized using an inverted microscope (Carl Zeiss AG, Oberkochen, Germany) and 10 images were captured for each group.

Total RNA extraction and real-time PCR

Total RNA was isolated from HASCs in the control and treatment groups by using trizol reagent, according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA in a 20- μ L reaction by using a PrimeScript RT Master Mix kit. Real-time reverse

transcription-PCR was performed with a SYBR Green PCR kit (Toyobo, Osaka, Japan) and ABI 7300 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers were designed as follows: human RUNX2 (forward, 5' -CCGCACAACCGCACCAT-3'; reverse, 5' -CGCTCCGGCCACAAAATCTC-3'), human OCN (forward, 5' -CATGAGAGCCCTCACA-3'; reverse, 5' -AGAGCGACACCCTAGAC-3'), human COL1 (forward, 5' -GGACACAATGGATTGCAAGG-3'; reverse, 5' -TAACCACTGCTCCACTCTGG-3'), human VEGF (forward, 5' -TAGTCGACATGAACTTTCTGCTG-3'; reverse, 5' -ATAAGCTTTCACCGCCTT-3') and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward, 5' -GGGCTGCTTTAACTCTGGT-3'; reverse, 5' -GCAGGTTTTTCTAGACGG-3'). Amplification and detection were performed under the following conditions: Incubation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and subsequent annealing and extension at 60°C for 34 sec. For each sample, GAPDH expression was analyzed to normalize target gene expression. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method[27]. Each sample was analyzed in triplicate.

Western blotting

After three washes with cold PBS, total protein was extracted from cells using lysis buffer. The proteins (10 µg) were resolved using 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% nonfat milk in PBS containing Tween-20 (PBS-T) for 2 h at room temperature. The membranes were incubated at 4°C overnight with primary antibodies specific for RUNX2 (1:1000), OCN (1:1000), COL1 (1:500), VEGFR1 (1:1000), Angiogenin (1:500), ERK1/2(1:500), p-ERK1/2 (1:500), p38 (1:1,000), p-p38 (1:1,000), JNK (1:500), and p-JNK (1:500). After three washes with PBST (0.5% Tween 20 in PBS), the membranes were incubated with the relevant secondary antibodies (1:1000) for 1 h at 37°C, washed and visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore) and visualized using the ImageQuantLAS 4000 mini imaging system (General Electrics, USA). Three independent trials of each experiment were carried out. β -actin (1:500) served as an internal control.

VEGF quantification and HUVECs tube formation assay

The culture supernatant of control and treatment groups was collected from the *in vitro* coculture system after 14 days and assayed to measure the level of VEGF. A human VEGF ELISA kit was used to quantify VEGF in medium from HASCs and HASCs/HAMSCs groups, according to the manufacturer's instructions. The measured values were expressed as fold changes over that of the control: HASCs treated without HAMSCs.

HUVECs were subjected to the culture supernatant from control and treatment groups to assay the formation of tube-like structures. Six-well culture plates were coated with Matrigel according to the manufacturer's instructions. After HUVECs were incubated in EBM containing 1% FBS and EGM-2 BulletKit for 6 h and plated onto the layer of Matrigel at a density of 5×10^4 cells/well, medium was replaced by the culture supernatant from HASCs and HASCs/HAMSCs groups. Matrigel cultures were incubated at 37°C for 24 h. Following rinsing with PBS, tube formation was visualized using an inverted microscope (Carl Zeiss AG, Oberkochen, Germany) and representative network of formed tube structures was randomly photographed five shots per each group.

Statistical analysis

All the quantitative results were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean \pm standard deviation from at least three separate experiments.

The Student's t-test was used to compare data between the two groups. A value of $p < 0.05$ was considered to be statistically significant.

Results

HAMSCs promoted HASCs proliferation

Cell cycle fractions (G0, G1, S, and G2M phases) were determined by flow cytometry at 1, 3, and 5 days to measure the proliferation of HASCs seeded in the transwell coculture system. There was no significant difference between the proliferation level of HAMSCs and HASCs. However, a statistically increase of S-phase checkpoints with HASCs: HAMSCs ratios in coculture groups compared with the single-culture groups was detected (Fig 1). Our previous studies have confirmed that HAMSCs were capable of enhancing HBMSCs proliferation[20]. The present results further showed that HAMSCs could also accelerate HASCs proliferation in the transwell coculture system.

HAMSCs promoted ALP activity and extracellular matrix mineralization in HASCs

To measure the positive effects of HAMSCs on osteogenic differentiation of HASCs, we investigated ALP activity and extracellular matrix mineralization in HAMSCs, HASCs, and HASCs/HAMSCs groups at different time points. We found that although HAMSCs osteogenesis was much lower than HASCs', the ALP activity gradually increased in the treatment groups with HASCs: HAMSCs ratio at day 7 and 14, indicating HAMSCs up-regulated the osteoblastic differentiation of HASCs (Fig 2A and 2C).

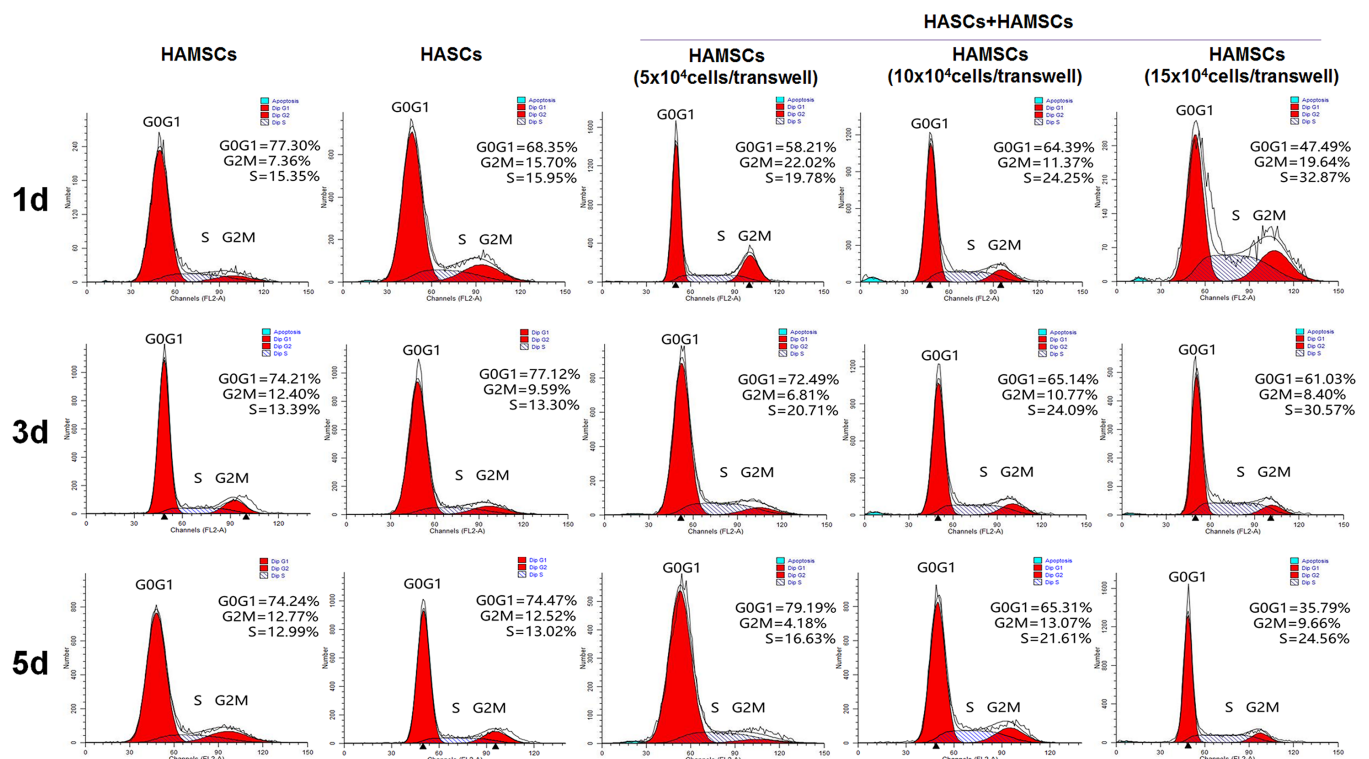


Fig 1. HAMSCs promoted HASCs proliferation. The cell cycle fractions (G0, G1, S, and G2 M phases) of HASCs cultured with or without HAMSCs were determined by flow cytometry at 1, 3 and 5 d.

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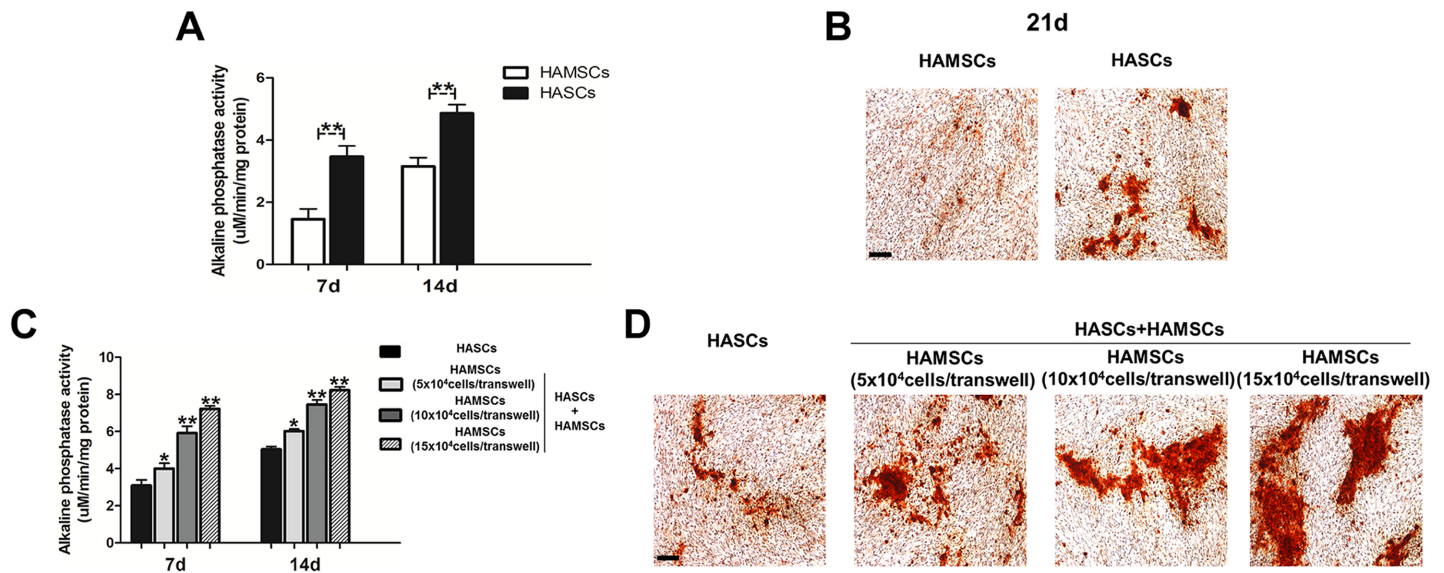


Fig 2. ALP activity and mineralized matrix deposition in HAMSCs, HASCs, and HASCs/HAMSCs groups. (A): ALP activity in HAMSCs and HASCs groups was measured at 7 and 14 d using ALP assay kit. (B): Mineralized matrix deposition in HAMSCs and HASCs groups was measured at 21 d by Alizarin red S. (C): ALP activity in HASCs and HASCs/HAMSCs groups was measured at 7 and 14 d using ALP assay kit. (D): Mineralized matrix deposition in HASCs and HASCs/HAMSCs groups was measured at 21 d by Alizarin red S. Scale bar: 300 μ m. *P < 0.05 and **P < 0.01 in contrast to the HAMSCs or HASCs groups.

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The HAMSCs, HASCs, and HASCs/HAMSCs culture surfaces stained positively for extra-cellular matrix was measured after 21 days. HASCs formed more mineralized matrix compared with HAMSCs. Moreover, increased level of mineralization in HASCs/HAMSCs groups was observed after 21 days in comparison with the HASCs single-culture groups. These observations demonstrated that HAMSCs positively promoted the mineralization in HASCs (Fig 2B and 2D).

HAMSCs promoted osteogenic and angiogenic markers expression in HASCs

Human RUNX2, OCN, and COL1 gene expression were analyzed by real-time PCR in HASCs after 14 days, with and without HAMSCs (Fig 3A, 3B and 3C). Significant higher levels of these osteogenic markers expression were observed in HASCs/HAMSCs groups with increased HASCs: HAMSCs ratio. VEGF, a valuable cytokine engaged in blood vessel formation and angiogenesis, was also up-regulated in HASCs cocultured with HAMSCs (Fig 3D). Western blotting showed the protein expression of RUNX2, OCN, COL1, VEGFR1, and Angiogenin were increased in HASCs/HAMSCs groups (Fig 3E). These results suggested HAMSCs enhanced osteogenic and angiogenic differentiation of HASCs.

HAMSCs promoted angiogenesis and ERK1/2 phosphorylation in HASCs

The effects of HAMSCs on angiogenesis in HASCs were further measured by VEGF ELISA assay of the culture medium on day 14 and tube formation assay of HUVECs at 24 h after culture. The HASCs/HAMSCs groups secreted significantly higher level of VEGF than HASCs groups, and the VEGF level gradually increased with HASCs: HAMSCs ratio (Fig 4A). The HUVECs tube formation assay demonstrated that the addition of culture supernatant from

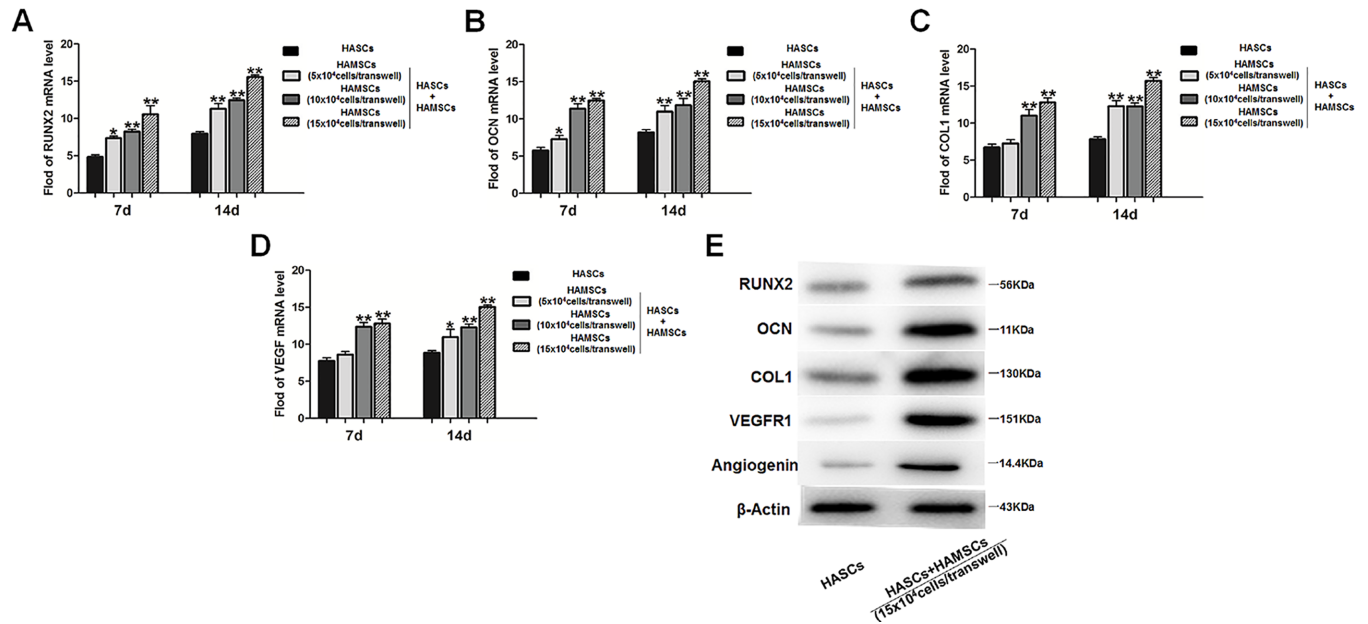


Fig 3. Expression of relative genes and proteins in HASCs cultured with or without HAMSCs. (A, B, C and D): The mRNA expression of RUNX2, OCN, COL1, and VEGF were analyzed by real-time PCR at 14 d. GAPDH were used as the internal control. (E): Protein expression of RUNX2, OCN, COL1, VEGFR1, and Angiogenin were determined by Western blotting at 14 d, β-actin served as an internal control. *P < 0.05 and **P < 0.01 in contrast to the HASCs groups.

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HASCs/HAMSCs groups significantly increased tube structures formed by HUVECs. Extensive vascular networks were observed and increased with HASCs: HAMSCs ratio in HUVECs subjected to culture medium from HASCs/HAMSCs system (Fig 4C). However, whether the

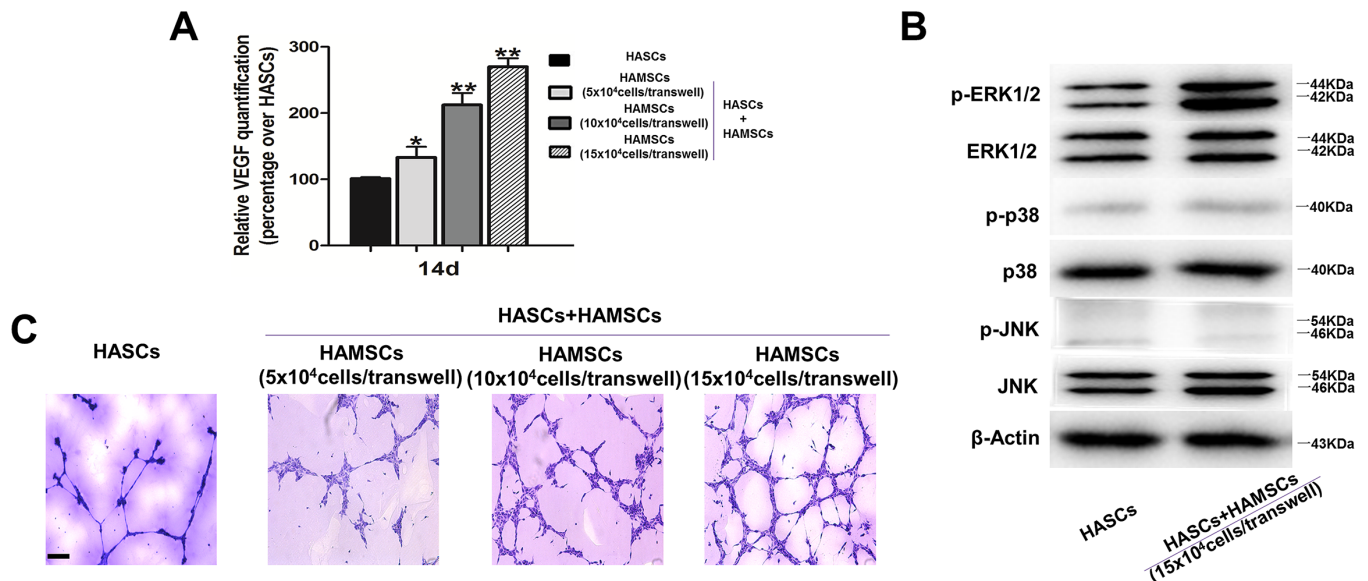


Fig 4. HAMSCs promoted angiogenesis and ERK1/2 phosphorylation in HASCs. (A): The VEGF level in culture supernatant from HASCs and HASCs/HAMSCs groups was measured by VEGF ELISA assay on day 14. (B): Tube formation from HUVECs was detected at 24 h after culture. (C): Protein expression of p-ERK, ERK, p-p38, p38, p-JNK, and JNK were determined by Western blotting at 14 d, β-actin served as an internal control. Scale bar: 300 μm. *P < 0.05 and **P < 0.01 in contrast to the HASCs groups.

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increased VEGF level is related to the interaction between HASCs and HAMSCs should be studied further.

Our previous studies have indicated that MAPK signal pathway is involved in the HAMSCs-driven osteogenic differentiation of HBMSCs [26]. In addition, it was also reported that MAPK signal pathway played an important role in regulating HASCs osteogenic differentiation [28]. Here, to explore the underlying mechanism, we investigated the effects of HAMSCs on MAPK activation in HASCs. In contrast to HASCs single culture groups, HAMSCs activated the ERK1/2 phosphorylation level in HASCs/HAMSCs groups, while neither the p38 nor JNK phosphorylation appeared to be involved (Fig 4B). These findings demonstrated that HAMSCs enhanced ERK1/2 phosphorylation, which might play a role in modulating HASCs osteogenesis and angiogenesis.

Discussion

The present study identified the valuable effects of HAMSCs on promoting osteogenic and angiogenic potential of HASCs *in vitro*. We found that HAMSCs promoted proliferation, drove osteogenic differentiation, and enhanced angiogenic via secretion increased VEGF in HASCs. Mechanismly, we demonstrated that ERK1/2-MAPK phosphorylation is associated with the osteogenesis and angiogenesis modulated by HAMSCs, while neither the p38 nor JNK phosphorylation appeared to be involved.

The application of MSCs has been widely designed to promote osteogenesis and angiogenesis for regenerating new bone formation in tissue engineering approaches. HBMSCs, osteoblasts (OB), and dental pulp stem cells (DPSCs) have been used [29–31], but most have disadvantages, such as limited availability, high immunogenicity and ethics problems. As an abundant source of stem/progenitor cells, adipose tissue became the focus of considerable interest in regenerative tissue engineering [32]. Among these cells, HASCs gained more attention in recent decades because they can be easily expanded and differentiated into a variety of cells along multiple lineages [33]. Since adipose tissue is abundant and easily access, HASCs exhibit enormous potential for clinical translation into regeneration therapies [34]. Therefore, how to effectively modulate the osteogenic and angiogenic differentiation of HASCs has become an emerging medical problem in bone tissue engineering [35–37].

HAMSCs, isolated from discarded human term placenta, exhibit a potential advantage over other types of MSCs [19]. Our previous findings have showed that HAMSCs were capable of promoting proliferation and osteogenic differentiation of HBMSCs [26], which led us to hypothesize the role of HAMSCs in HASCs-involved new bone formation. The S-phase checkpoints evaluated in HAMSCs, HASCs, and HASCs/HAMSCs groups at 1, 3, and 5 days suggested that HAMSCs promoted HASCs proliferation at early-stage of differentiation. ALP is an early marker of the osteogenic differentiation. Although HAMSCs osteogenesis was much lower than HASCs', treatment of HAMSCs remarkably up-regulated the ALP activity in HASCs. Increased mineralized nodule formation and calcium deposition activity in HASCs cocultured with HAMSCs indicated the positive effects of HAMSCs on late-stage of osteogenic differentiation [38–40]. RUNX2 is a key transcriptional factor associates with several transcription factors and binds to specific nuclear matrix [41]. It is capable of integrating a variety of organize crucial events during the early-stage osteoblastic differentiation of MSCs [42]. OCN is a marker of mature osteogenesis phenotype reflecting calcium deposition during the late-stage osteoblastic differentiation [43]. COL1 has been extensively investigated as the most important structural protein of natural bone [44], and its main organic component has been shown to display excellent osteoconductive properties *in vitro* and *in vivo* [45, 46]. Collectively, the real-time PCR analysis of RUNX2, OCN, and COL1 gene expression suggested HAMSCs

motivated HASCs osteogenic differentiation in both early- and late-stage. Additionally, protein expression showed the same variation, which helped us better comprehend HAMSCs' mechanism for action in HASCs-involved bone regeneration.

Bone is a vital organism that needs intraosseous vasculature to maintain normal metabolism [47]. A number of studies have demonstrated that rapid revascularization secrete osteogenic factors is necessary for initiating reliable bone formation in bone regeneration [48, 49]. Thus, developing proangiogenic molecules is becoming a key factor in tissue repair. HASCs can secrete a broad range of angiogenic factors, and share equal angiogenic capacity compared with HBMSCs [50]. HAMSCs are capable of acting as stromal cells in the formation, stabilization, and maturation of newly formed vessel [51, 52]. Therefore, we hypothesize the effects of HAMSCs in HASCs-involved neovascularization. VEGF, a cytokine engaged in angiogenesis and osteogenesis, evidently enhances new bone formation in animal fracture models [53, 54]. In this study, higher level of VEGF secretion was found in HASCs/HAMSCs groups than HASCs groups. HUVECs, the most commonly used type of cells for vessel regeneration studies [55], formed abundant vascular networks by treatment with culture supernatant from HASCs/HAMSCs groups. In addition, angiogenesis related gene and protein expression also indicated the positive effects of HAMSCs. Thus, these results support HAMSC as activator in HASCs-involved angiogenesis.

To unveil the underlying molecular mechanisms by which HAMSCs promote HASCs osteogenic and angiogenic differentiation, MAPK signaling, the activation of which is a crucial trigger of MSCs differentiation [56–58], was investigated. Phosphorylation is a remarkable mechanism that modulates the initiating of various factors during osteogenesis and angiogenesis [59–61]. Our previous study determined that ERK1/2 phosphorylation was important for the HAMSCs-driven osteogenic differentiation of HBMSCs [62]. Interestingly, the present study also suggested the p-ERK1/2 level was up-regulated by HAMSCs in HASCs. Previous study reported the ERK1/2-dependent RUNX2 activation was an important regulatory mechanism of osteoblast differentiation [63]. In view of the expression of p-ERK1/2 and RUNX2 were both increased by HAMSCs, the ERK1/2-RUNX2 signaling may represent a suitable therapeutic target for promoting HASCs-involved bone regeneration.

The present study first demonstrates the influence of cocultured HAMSCs in enhancing the osteogenic and angiogenic differentiation of HASCs as well as the potential signal pathway. HAMSCs added to HASCs promoted proliferation, osteogenesis and angiogenesis *in vitro* at the increased HASCs: HAMSCs ratios. The ERK1/2 phosphorylation level, RUNX2 expression, and VEGF secretion are involved in the underlying mechanism. These findings shed light on the key characteristics of HAMSCs and expand the application of HASCs in bone defects reconstruction.

Supporting information

S1 File. Western blotting in Figs 3 and 4.
(ZIP)

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Author Contributions

Data curation: Lidong Yu.

Investigation: Chunli Zhang, Lidong Yu, Songjian Liu.

Validation: Songjian Liu.

Writing – original draft: Chunli Zhang.

Writing – review & editing: Yuli Wang.

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