Human amniotic epithelial cells regulate osteoblast differentiation through the secretion of TGFβ$_1$ and microRNA-34a-5p

GUILING WANG[1,2], FENG ZHAO[2], DI YANG[1], JING WANG[3], LIHONG QIU[1] and XINING PANG[2]

[1]Department of Endodontics, School of Stomatology, China Medical University, Shenyang, Liaoning 110002; [2]Department of Stem Cells and Regenerative Medicine, Key Laboratory of Cell Biology, Ministry of Public Health of China, China Medical University, Shenyang, Liaoning 110013; [3]Department of Anal and Intestinal Surgery, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

Received April 20, 2017; Accepted October 20, 2017

DOI: 10.3892/ijmm.2017.3261

Abstract. Since the beginning of the use of stem cells in tissue regenerative medicine, there has been a search for optimal sources of stem cells. Human amniotic epithelial cells (hAECs) are derived from human amnions, which are typically discarded as medical waste, but were recently found to include cells with trilineage differentiation potential *in vitro*. Previous study has focused on the osteogenic differentiation ability of hAECs as seed cells in bone regeneration; however, their paracrine effects on osteoblasts (OBs) are yet to be elucidated. In the present study, conditioned medium (CM) derived from hAECs was used to determine their paracrine effects on the human fetal OB cell line (hFOB1.19), and the potential bioactive factors involved in this process were investigated. The results suggested that hAEC-CM markedly promoted the proliferation, migration and osteogenic differentiation of hFOB1.19 cells. Expression of transforming growth factor β$_1$ (TGFβ$_1$) and microRNA 34a-5p (miR-34a-5p) were detected in hAECs. Furthermore, it was demonstrated that TGFβ$_1$ and miR-34a-5p stimulated the differentiation of hFOB1.19 cells, and that TGFβ$_1$ promoted cell migration. Moreover, the effects of hAEC-CM were downregulated following the depletion of either TGFβ$_1$ or miR-34a-5p. These results demonstrated that hAECs promote OB differentiation through the secretion of TGFβ$_1$ and miR-34a-5p, and that hAECs may be an optimal cell source in bone regenerative medicine.

Correspondence to: Dr Lihong Qiu, Department of Endodontics, School of Stomatology, China Medical University, 117 Nanjing North Street, Heping, Shenyang, Liaoning 110002, P.R. China
E-mail: drqlh@yahoo.com

Dr Xining Pang, Department of Stem Cells and Regenerative Medicine, Key Laboratory of Cell Biology, Ministry of Public Health of China, China Medical University, 77 Puhe Street, Shenbei New District, Shenyang, Liaoning 110013, P.R. China
E-mail: pangxining@126.com

Abbreviations: hAECs, human amniotic epithelial cells; hAMSCs, human amniotic mesenchymal stem cells; OB, osteoblast; OC, osteoclast; TGFβ$_1$, transforming growth factor β$_1$; miR-34a-5p, microRNA-34a-5p; hFOB1.19, human fetal OB cell line; CM, conditioned medium; ESC, embryonic stem cell; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; Pnpp, para-nitrophenyl phosphate; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; OD, optical density; PBS, phosphate-buffered saline; $A_{initial/final}$, initial/final absorbance; CON, control

Key words: human amniotic epithelial cells, human amniotic mesenchymal stem cells, osteoblast, transforming growth factor β$_1$, microRNA-34a-5p

Introduction

Stem cells have provided promising therapeutic applications with regard to biological and functional restoration of tissue defects; however, optimal sources of stem cells remain to be determined. Despite their high proliferative capacity and totipotency, embryonic stem cells (ESCs) are not used in stem cell therapy due to ethical concerns and tumorigenic risk. As an alternative, adult stem cells are widely considered as an acceptable cell source in bone regenerative medicine (1); however, their applications thus far have been restricted due to the invasive isolation procedure and limited sources. In recent years, cells derived from the amnion have attracted increasing attention as an appropriate source due to the relatively easy isolation procedure and lack of ethical concerns.

The amnion develops from the epiblast by the 8th day of fertilization and prior to gastrulation, which provides it with the combined qualities of both embryonic and adult stem cells. Human amniotic epithelial cells (hAECs) have been shown to possess trilineage differentiation potential *in vitro* in the endoderm (liver and pancreas), mesoderm [cardiomyocytes and osteoblasts (OBs)], and ectoderm (neural cells) (2,3). Furthermore, hAECs have been previously found to exert positive effects on immunoregulation (4,5) and tissue regeneration (6-8). As regards the applications of hAECs in bone regeneration, previous studies have focused on their osteogenic differentiation ability as seed cells (3,9,10). In recent years, researchers have suggested that stem cells may exert their regenerative effects primarily in a paracrine manner (11-13);

precursor cells in the host may be recruited to bone defects and induced to accelerate the regeneration of bone tissue by secretions from the stem cells (14,15). However, the paracrine effects of hAECs in bone regeneration are yet to be elucidated.

OBs interact with osteoclasts (OCs) *in vivo* to maintain bone homeostasis. Imbalance between OB-mediated bone formation and OC-mediated bone resorption may be triggered by surrounding stimuli and may result in a series of pathological bone disorders, including osteopenia, osteoporosis, periodontitis and arthritis. Therefore, the viability of OBs is crucial for the maintenance of bone remodeling and regeneration. The aim of the present study was to investigate the effects of conditioned medium (CM) from hAECs on the function of the human fetal OB cell line (hFOB1.19). The results suggested that the function of hFOB1.19 cells was markedly promoted by hAEC-CM. Additionally, transforming growth factor β_1 (TGFβ_1) and microRNA-34a-5p (miR-34a-5p) were found to be expressed in the hAECs. TGFβ_1 is secreted as a soluble factor into the medium, while miR-34a-5p is likely to be enclosed in extracellular vesicles (16,17). The role of these two factors in the potential paracrine effects of hAECs was further investigated to determine whether hAECs can regulate the differentiation of OBs through TGFβ_1 and miR-34a-5p.

Materials and methods

Isolation and culture of cells. The present study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Human amnions were obtained, with written informed consent, from healthy mothers undergoing cesarean section. All the patients were negative for human immunodeficiency virus-1, hepatitis B and hepatitis C virus infection. The human amnion layer was mechanically peeled away from the placenta and rinsed with phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin solution. The layer was then cut into ~25-cm^2 pieces with scissors, and the chorion and residual blood clots were removed with tweezers. Subsequently, each piece was incubated with 10 ml 0.25% trypsin solution (Gibco; Thermo Fisher Scientific, Carlsbad, CA, USA) at 37˚C for 20, 10 and 5 min, sequentially, to isolate hAECs. Trypsin was inactivated by the addition of 1 ml heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA). Supernatant was collected and filtered through a cell sieve, and the filtrate was centrifuged at 1,000 x g for 5 min. The resulting cell pellet was resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (HyClone) supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 1% GlutaMAX, 1% non-essential amino acids (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (defined as complete hAEC medium) in a humidified incubator at 37˚C with 5% CO_2. Unattached cells were removed 24 h later and the remaining cells were defined as passage 0 (P0). Cells were trypsinized and subcultured at a ratio of 1:2 upon reaching a confluence of 80-90%. hAECs at P2-P3 were used for subsequent assays.

To obtain human amniotic mesenchymal stem cells (hAMSCs), the remaining amnion was cut into small pieces and digested in 1 mg/ml collagenase (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA) diluted in DMEM/F12 for ~20 min, until only a small amount of amnion was visible. The supernatant was collected as described above and cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin.

The human fetal OB cell line hFOB1.19 was purchased from the Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM/F12 supplemented with 10% FBS and 0.3 mg/ml G418 (Sigma-Aldrich; Merck KGaA) at 33.5˚C in a 5% CO_2 atmosphere.

Flow cytometric analysis. hAECs at P3 were harvested with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and washed with PBS, and then ~1x10^6 cells were resuspended in 100 μl cold PBS together with the following monoclonal phycoerythrin-conjugated anti-human primary antibodies (5 μl per test in 100 μl PBS; all purchased from BioLegend, San Diego, CA, USA): Anti-CD44 (cat. no. 338807), anti-CD90 (cat. no. 32810), anti-CD105 (cat. no. 323205), anti-CD117 (cat. no. 313204), anti-SSEA-4 (cat. no. 330405) and anti-HLA-DR (cat. no. 307605). Cells incubated in PBS were used as a blank control. After incubation in the dark for 30 min at 4˚C, hAECs were washed with cold PBS and assessed for the expression of stem cell-related cell surface markers by flow cytometry (BD Biosciences, San Jose, CA, USA). A minimum of 10,000 cell events were acquired per assay.

Differentiation of hAECs in vitro. To induce osteogenic differentiation, hAECs were treated with 10^{-5} mM dexamethasone (Ryon Biological Technology, Shanghai, China), 10 mM β-glycerophosphate and 50 mg/l ascorbic acid in complete hAEC medium, with replenishment of the medium every 3 days. Following osteogenic induction for 6 and 21 days, the cells were stained with an alkaline phosphatase (ALP) staining kit (Beyotime Institute of Biotechnology, Shanghai, China) and 40 mM Alizarin Red S (pH 4.2; Sigma-Aldrich; Merck KGaA) solution. For neurogenic induction, hAECs were pre-induced with 10 μg/ml basic fibroblast growth factor (Thermo Fisher Scientific, Inc.) in complete hAEC medium for 24 h, then cultured with 1 μM all-trans retinoic acid (Sigma-Aldrich; Merck KGaA) in DMEM/F12 (without FBS) for another 24 h, and cellular immunofluorescent staining was conducted to detect the expression of β-tubulin and nestin, as markers of neurogenic differentiation. Pancreatic differentiation was induced with 10 mM nicotinamide (Sigma-Aldrich; Merck KGaA) in complete hAEC medium for 21 days, followed by immunofluorescent staining of cells to detect expression of the pancreatic differentiation markers glucagon and pancreatic polypeptide.

Cell immunofluorescent staining. hAECs were fixed with 4% paraformaldehyde solution for 30 min at room temperature and washed twice with PBS. After permeabilization in 0.1% Triton X-100 in PBS for 20 min followed by blocking in 3% bovine serum albumin for 45 min, the cells were incubated with the following primary antibodies (all purchased from Abcam, Cambridge, MA, USA): Anti-β-tubulin (cat. no. ab28035; mouse anti-human; 1:200), anti-nestin (cat. no. ab22035; mouse anti-human; 1:200), anti-glucagon (cat. no. ab10988; mouse anti-human; 1:200) and anti-pancreatic polypeptide (cat. no. ab77192; goat anti-human; 1:150)

in a humidified box at 4˚C. On the following day, the cells were washed three times in PBS and incubated with Alexa Fluor® 488-conjugated secondary antibodies (antibodies 1 and 2) for 1 h at room temperature. Antibody 1 (cat. no. ab150129; donkey anti-goat; 1:500) was used to detect pancreatic polypeptide and antibody 2 (cat. no. ab150113; goat anti-mouse; 1:500) was used to detect the other proteins. This and subsequent processes were protected from light. After three washes in PBS, cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology) diluted in PBS (1:1,000) for 5 min, and the cells were then washed in PBS and photographed with an immunofluorescent microscope (Carl Zeiss, Oberkochen, Germany).

Collection of CM. hAECs were cultured in complete hAEC medium, and when the cells reached a confluence of 80-90% (~1x10^6 cells per 25 cm^2 flask), the medium was replaced with DMEM/F12 supplemented with 10% FBS (defined as complete medium). After 24 h, the medium was collected and centrifuged at 1,500 x g for 5 min to remove cell debris, and the supernatant was sub-packaged and stored at -80˚C. The CM used in subsequent assays was at a final concentration of 50% (supernatant mixed with fresh complete medium at a 1:1 ratio).

Cell proliferation assay. hFOB1.19 cells were seeded at density of 5x10^3 cells/well into a 96-well plate. After 24 h, the cells were treated with 200 μl CM for 3 days. Cells treated with the standard complete medium served as a control (CON). Cell proliferation was subsequently measured with an MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation assay; Promega, Madison, WI, USA) according to the manufacturer' s instructions. After 3 days, the medium in each well was replaced with 100 μl serum-free DMEM/F12 mixed with 20 μl MTS. After incubation in a CO_2 incubator for 3 h at 37˚C, the optical density (OD) of each well was measured at a wavelength of 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader and normalized to a blank control without cells.

Transwell migration assay. hFOB1.19 cells were seeded into the upper chambers of a Transwell system (pore size, 8 μm; Corning Costar, New York, NY, USA) at a density of 1x10^5 cells/well in 100 μl serum-free DMEM/F12, and 600 μl CM was added to the lower chamber. Cells treated with complete medium alone served as control (CON). Following incubation for 2 h, the cells were fixed in methanol for 3 min, then stained with hematoxylin and eosin. Non-migrated cells in the upper chamber were removed with cotton swabs, and the numbers of migrated cells were counted in five randomly selected images captured with an inverted microscope.

Osteogenic differentiation of hFOB1.19. hFOB1.19 cells were seeded at density of 1x10^5 cells/well into a 6-well plate. When the cells reached a confluence of ~90%, they were cultured in designed medium and supplemented with 10 mM β-glycerol phosphate (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) and 50 mg/l ascorbic acid (Ryon Biological Technology) at a culture temperature of 37˚C. After 6 days, osteogenic differentiation was assessed by ALP activity and fluorescent quantitative polymerase chain reaction (qPCR).

ALP activity. ALP activity was measured with an ALP Yellow [para-nitrophenyl phosphate (pNPP)] Liquid Substrate System for ELISA (Sigma-Aldrich; Merck KGaA), as previously described (18). In brief, hFOB1.19 cells were washed twice and lysed with RIPA lysis buffer containing proteinase inhibitor. A total of 10 μl cell lysate and 90 μl pNPP were mixed in a 96-well plate and the absorbance (A) at 405 nm was measured immediately (defined as $A_{initial}$) and again after 30 min at 37˚C (defined as A_{final}). A blank control (pNPP + RIPA buffer) was also measured. Protein concentration (mg/ml) was measured with a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Enzyme activity was calculated using the following formula: ALP activity (U/ml) = [(A_{final} - $A_{initial}$) x R x 10]/18.45, with 18.45 being the extinction coefficient, and R the dilution factor divided by the path length (for a conventional 96-well plate and a reaction volume of 100 ml, the path length was ~0.31 cm). The calculated ALP activity was subsequently normalized to the protein amount.

RNA extraction and gene expression analysis. Total RNA was isolated using TRIzol reagent (Takara, Dalian, China) according to the manufacturer' s protocol, and RNA concentration was quantified with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA with an A260/A280 ratio of 1.8-2.0 was considered to be pure.

To evaluate the expression of osteogenic differentiation markers, 1 μg total RNA was reverse transcribed with a PrimeScript™ reverse transcription (RT) reagent kit with gDNA Eraser (Takara), according to the manufacturer's instructions. Fluorescent qPCR was performed with SYBR Premix Ex Taq™ II (Takara) in an ABI-PRISM 7500 system (Applied Biosystems, Foster City, CA, USA). The relative expression levels of the target genes were normalized to the expression of β-actin using the ΔΔCq method. The primer sequences used were as follows: ALP, 5'-CCAAGGACGCTGGGAAATCT/TATGCATGAGC TGGTAGGCG-3'; osteocalcin (OCN), 5'-GACGAGTTGG CTGACCACA/CAAGGGGAAGAGGAAAGAAGG-3'; osteopontin (OPN), 5'-GATGAATCTGATGAACTGGTCACT/ GGTGATGTCCTCGTCTGTAGCA-3'; runt-related transcription factor 2 (RUNX2), 5'-TAGGCGCATTTCAGGTG CTT/ GGTGTGGTAGTGAGTGGTGG-3'; and β-actin, 5'-AGGA TTCCTATGTGGGCGAC/ATAGCACAGCCTGGA TAGCAA-3'.

To evaluate the expression of miR-34a-5p, 5 μg total RNA isolated from hAECs, hAMSCs and hFOB1.19 cells was subjected to poly(A) tailing with a Poly(A) Tailing kit (Applied Biosystems), and the tailed RNA was further extracted and purified with phenol-chloroform. DNA digestion, RT and qPCR were then conducted as mentioned above. The relative expression level of miR-34a-5p was normalized to the expression of U6 using the ΔΔCq method. The primer sequences used were as follows: RT primer 1, 5'-GCTGTCAACGATACGCTACGTAACGGC ATGACAGTGTTTTTTTTTTTTTTTTTTA-3'; RT primer 2, 5'-GCTGTCAACGATACGCTACGTAACGGCATG ACAGTGTTTTTTTTTTTTTTTTTTG-3'; RT primer 3, 5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG TTTTTTTTTTTTTTTTTTTC-3'; hsa-miR-34a-5p, 5'-TGG CAGTGTCTTAGCTGGTTGT/GCTGTCAACGATACGCTA CGT-3'; and U6, 5'-GCTGTCAACGATACGCTACGT/TTC

ACGAATTTGCGTGTCAT-3'. RT primers 1-3 were mixed in a 1:1:1 ratio to serve as a replacement RT primer mix in the PrimeScript™ RT reagent kit with gDNA Eraser during RT.

ELISA. The supernatants isolated from hAECs as mentioned above were analyzed for the expression of $TGF\beta_1$ using ELISA. The concentration of $TGF\beta_1$ was measured with a Human Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

$TGF\beta_1$ assays. The effects of $TGF\beta_1$ on the function of hFOB1.19 cells were evaluated by treating cells with 5 ng/ml recombinant human $TGF\beta_1$ protein (PeproTech, Rochy Hill, NJ, USA) in complete medium. Cell proliferation was then assessed as above.

In a separate assay, $TGF\beta_1$ contained in the hAEC-CM was neutralized through overnight incubation with 5 μg/ml human $TGF\beta_1$ antibody (R&D Systems).

The effects of exogenous $TGF\beta_1$ or $TGF\beta_1$ neutralization on the migration and differentiation of hFOB1.19 cells were subsequently assessed via the aforementioned Transwell, qPCR and ALP activity assays.

Cell transfection. hAECs (~$2.5x10^5$ cells/well) and hFOB1.19 cells (~$1x10^5$ cells/well) were plated into 6-well plates to ensure that a confluence of ~70-80% was reached. On the following day, hFOB1.19 cells were transfected with 100 nM miR-34a-5p mimics and hAECs were transfected with 100 nM miR-34a-5p inhibitor (all from GenePharma, Shanghai, China) using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions; miR-NC and miR-NC inhibitor were used as negative control, respectively. The medium was replaced after 6 h, and the efficiency of transfection was determined by qPCR 3 days later. Osteogenicity was also assessed via the aforementioned differentiation assays following transfection. Furthermore, the proliferative and migratory abilities of cells transfected with miR-34a-5p mimics were assessed as described above. All assays were performed 3 days after transfection.

hAECs were also transfected with Cy3-conjugated miR-34a-5p mimics, as mentioned above. The fluorescence of the cells was subsequently observed and photographed using an immunofluorescent microscope (Carl Zeiss, Oberkochen, Germany) after cells were washed with PBS.

Statistical analysis. Data are expressed as mean ± standard deviation. Comparisons between groups were performed using an independent samples t-test. Comparisons among ≥3 groups were performed using one-way analysis of variance and a Bonferroni post hoc test. All statistical analyses were performed with SPSS 13.0 software and a P-value <0.05 was considered to indicate statistical significance. All assays were repeated three times.

Results

Characteristics of hAECs. Attachment of the hAECs to the plastic dishes was observed on the day after their isolation from amnion tissues, and the primary adherent cells typically reached full confluence after culture for 2-3 days. The hAECs assumed a cobblestone-like morphology (Fig. 1A), and the proliferation of hAECs was evident by P3.

The results of flow cytometric analysis demonstrated that the hAECs expressed the mesenchymal stem cell markers CD44, CD90 and CD105, and the ESC marker SSEA-4, suggesting pluripotency. The hematopoietic progenitor cell marker CD117 and the immune-related marker HLA-DR were not expressed (Fig. 1B), indicating that cells from the umbilical cord blood had not contaminated the hAEC isolates.

Differentiation of the hAECs was induced *in vitro* under appropriate conditions. Pancreatic, osteogenic and neurogenic differentiation were induced successfully (Fig. 1C), indicating the trilineage differentiation potential of hAECs towards endodermal, mesodermal and ectodermal lineages, respectively.

Effects of hAEC-CM on the function of hFOB1.19 cells in vitro. When hFOB1.19 cells were cultured in hAEC-CM for 3 days, the corresponding OD value was significantly higher compared with that of the CON group (Fig. 2A), suggesting that the proliferation of hFOB1.19 cells was markedly enhanced. Additionally, the effect of hAEC-CM on the migration of hFOB1.19 cells was detected in a Transwell system, and it was observed that hAEC-CM significantly accelerated the migration of cells after 2 h (Fig. 2B). Furthermore, the expression levels of ALP, OCN and OPN, as osteogenic differentiation-related markers, and of RUNX2, a specific transcription factor in ossification, were detected by qPCR. The results revealed that the expression levels of ALP, OCN, OPN and RUNX2 in hFOB1.19 cells were markedly increased by hAEC-CM (Fig. 2C). Differences in the activity of ALP were also confirmed with a pNPP assay (Fig. 2D). Collectively, these data indicated that hAEC-CM effectively promoted the function of hFOB1.19 cells.

Role of $TGF\beta_1$ in the effects of hAEC-CM. It was demonstrated by ELISA that the concentration of $TGF\beta_1$ was significantly higher in hAEC-CM compared with that in complete (CON) medium (Fig. 3A), indicating that $TGF\beta_1$ may contribute to the paracrine effects of hAECs. The effects of $TGF\beta_1$ on the function of hFOB1.19 cells were subsequently assessed with recombinant human $TGF\beta_1$ protein (5 ng/ml; PeproTech). After hFOB1.19 cells were incubated with $TGF\beta_1$ for 2 days, the OD value was 0.5237±0.0100, which was significantly lower compared with that of the CON group (0.6207±0.0165) (Fig. 3B). In addition, the number of migrated cells in the Transwell system was markedly elevated by $TGF\beta_1$ after 6 h (Fig. 3C). Furthermore, following osteogenic induction for 6 days, the relative mRNA expression levels of ALP and RUNX2 were significantly increased by $TGF\beta_1$ (Fig. 3Ea); however, the change in ALP activity was not statistically significant (P=0.147) (Fig. 3Eb).

Human $TGF\beta_1$ antibody (5 μg/ml; R&D Systems) was subsequently used to neutralize $TGF\beta_1$ in the hAEC-CM. The migration (Fig. 3D) and osteogenic differentiation (Fig. 3F) of hFOB1.19 cells were both attenuated when $TGF\beta_1$ was depleted. In conclusion, $TGF\beta_1$ may contribute to the stimulatory effects of hAEC-CM on the migration and osteogenic differentiation of hFOB1.19 cells.

Role of miR-34a-5p in the effects of hAEC-CM. The expression levels of miR-34a-5p were higher in hAECs compared with human amniotic mesenchymal stem cells and hFOB1.19 cells,

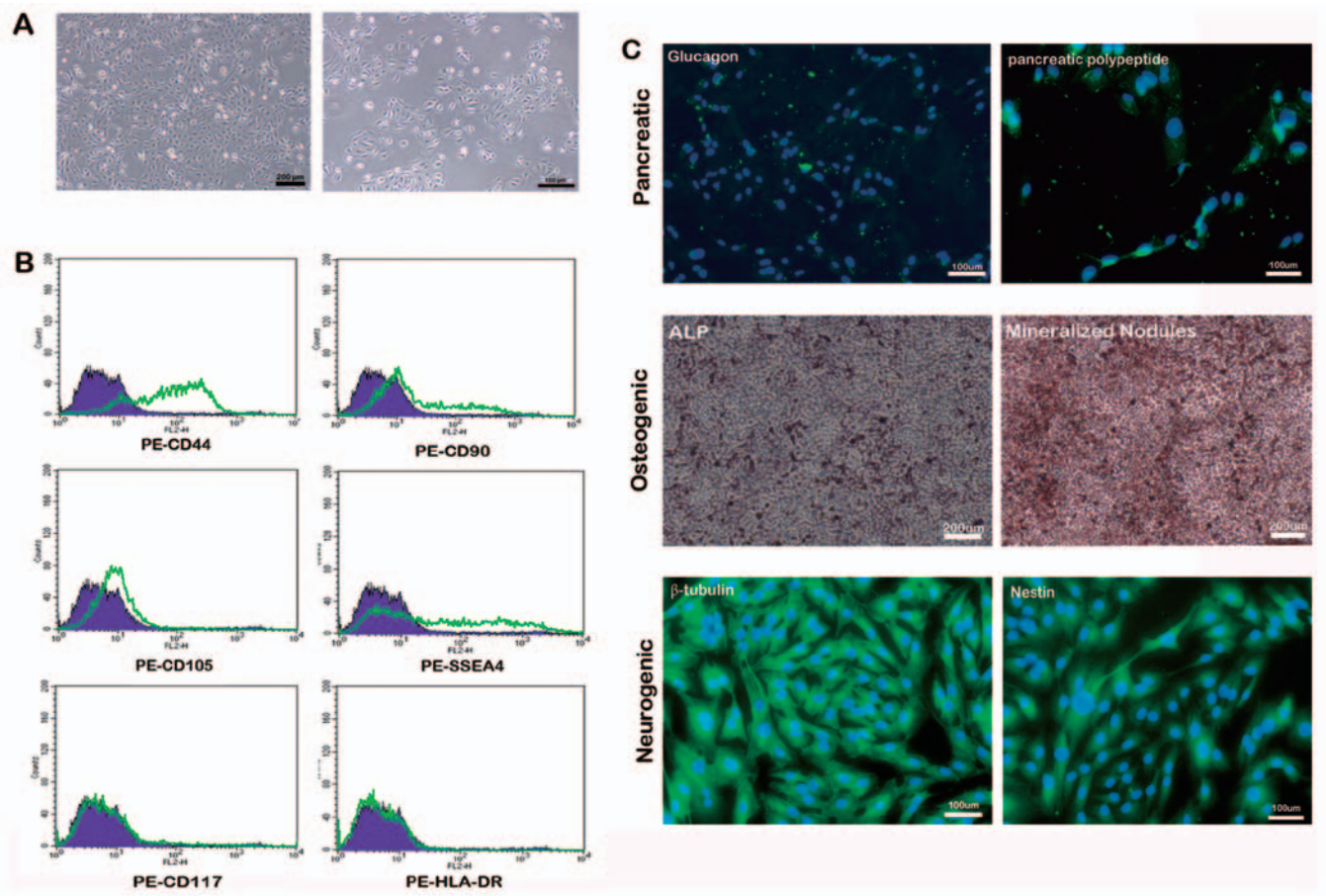

Figure 1. Characterization of hAECs isolated from amnion tissues. (A) Representative images of hAECs. (B) Flow cytometric analysis of stem cell-related cell surface marker expression on hAECs. (C) Pancreatic, osteogenic and neurogenic differentiation of the hAECs *in vitro*. hAECs, human amniotic epithelial cells; ALP, alkaline phosphatase.

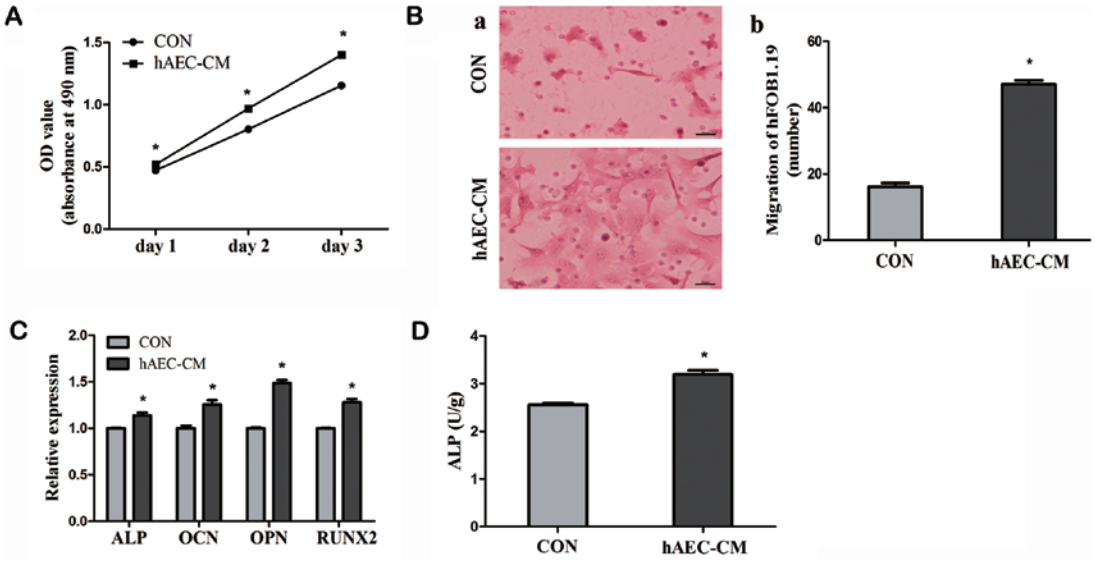

Figure 2. Effects of hAEC-CM on the function of hFOB1.19 cells. (A) Effect of hAEC-CM on the proliferation of hFOB1.19 cells. (B) (a) Representative images of migrated hFOB1.19 cells after culture with hAEC-CM in a Transwell system for 2 h; (b) graph showing the number of migrated hFOB1.19 cells. (C and D) Osteogenic differentiation of hFOB1.19 cells after culture in hAEC-CM for 6 days. *P<0.05. hAEC-CM, human amniotic epithelial cell-conditioned medium; hFOB1.19, human fetal osteoblast cell line; OD, optical density; CON, control; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor 2.

as measured by qPCR (Fig. 4A). The levels of miR-34a-5p also increased time-dependently during the differentiation of hFOB1.19 cells (Fig. 4B), suggesting that miR-34a-5p expression is positively associated with the differentiation of OBs.

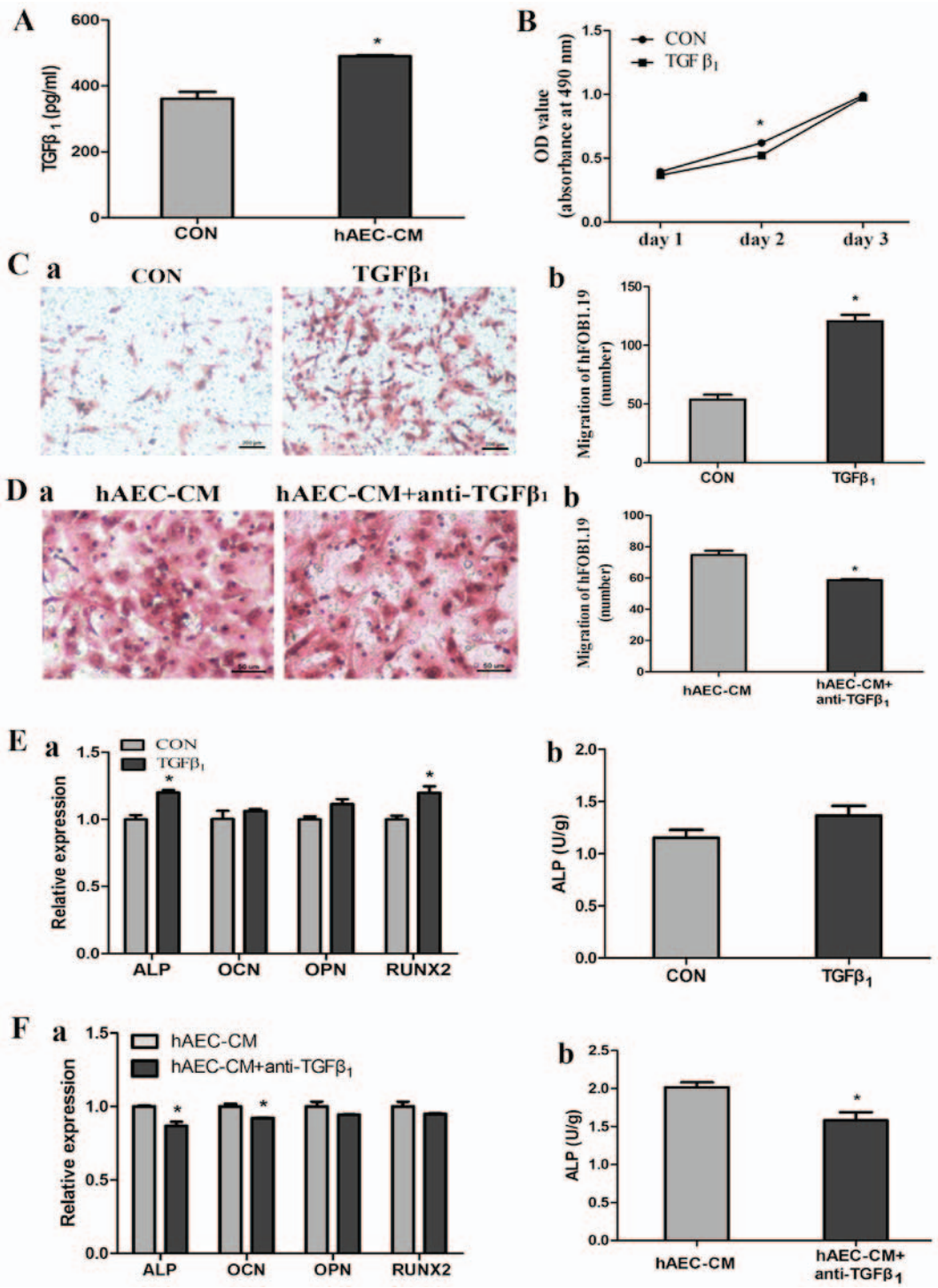

Figure 3. Role of $TGF\beta_1$ in the effects of hAEC-CM. (A) Concentration of $TGF\beta_1$ in the supernatant of hAECs and complete (control) medium. (B) Effect of 5 ng/ml $TGF\beta_1$ on the proliferation of hFOB1.19 cells. (C and D) Migration of hFOB1.19 cells after culture with 5 ng/ml $TGF\beta_1$ for 6 h (C) or hAEC-CM neutralized with 5 μg/ml $TGF\beta_1$ antibody for 2 h (D) in a Transwell system. (E and F) Osteogenic differentiation of hFOB1.19 cells after culture with (Ea and b) 5 ng/ml $TGF\beta_1$ or (Fa and b) hAEC-CM neutralized with 5 μg/ml $TGF\beta_1$ antibody. *P<0.05. TGF, transforming growth factor; hAECs, human amniotic epithelial cells; CM, conditioned medium; hFOB1.19, human fetal osteoblast cell line; OD, optical density; CON, control; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor 2.

hAECs were subsequently transfected with Cy3-conjugated miR-34a-5p mimics, to assess whether the miR-34a-5p mimics in the CM could be transferred into adjacent hFOB1.19 cells. Fluorescence signals were detected in the hFOB1.19 cells following incubation in the CM for 1 day (Fig. 4C), verifying that miR-34a-5p can be transported into the surrounding medium and transferred into adjacent cells.

miR-34a-5p mimics were transfected into hFOB1.19 cells to determine the specific roles of miR-34a-5p in the modulation of OB function. hFOB1.19 cells transfected with miR-NC were used as a negative control (Fig. 4D). Cell proliferation was downregulated in the miR-34a-5p mimics group (Fig. 4E), and the migration of cells was also markedly inhibited by miR-34a-5p mimics after 6 h (Fig. 4F). By contrast, osteogenic

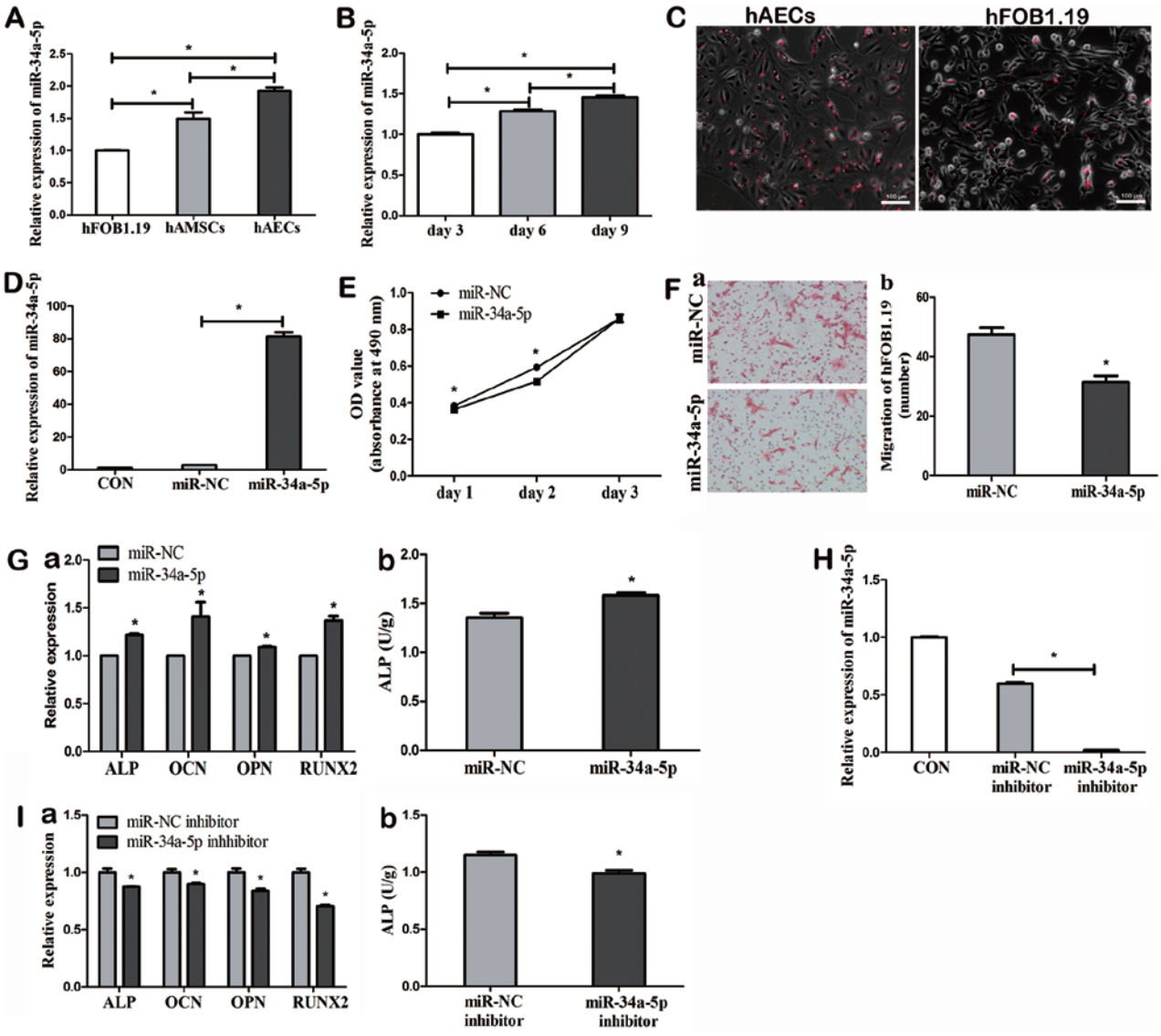

Figure 4. Role of miR-34a-5p in the effects of hAEC-CM. (A) Comparison of miR-34a-5p expression levels in hAECs, hAMSCs and hFOB1.19 cells. (B) Expression of miR-34a-5p during the osteogenic differentiation of hFOB1.19 cells. (C) Fluorescence images of hAECs transfected with Cy3-conjugated miR-34a-5p mimics (left), and hFOB1.19 cells after incubation with hAEC-CM for 1 day (right). (D-G) hFOB1.19 cells were (D) transfected with miR-34a-5p mimics, and the effects of miR-34a-5p on the (E) proliferation, (F) migration and (G) differentiation of hFOB1.19 cells were detected. (H and I) hAECs were (H) transfected with miR-34a-5p inhibitor, and (I) the effect of the hAEC-derived CM on the osteogenic differentiation of hFOB1.19 cells was detected. *P<0.05. hAECs, human amniotic epithelial cells; CM, conditioned medium; hFOB1.19, human fetal osteoblast cell line; hAMSCs, human amniotic mesenchymal stem cells; OD, optical density; CON, control; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor 2; NC, negative control.

differentiation was significantly enhanced (Fig. 4G), suggesting that miR-34a-5p only stimulates the differentiation of OBs.

hAECs were also transfected with miR-34a-5p inhibitor, which led to a significant decrease in the expression levels of miR-34a-5p (Fig. 4H). Furthermore, the pro-differentiation effect of hAEC-CM on hFOB1.19 cells was weakened, as indicated by reduced expression levels of the osteogenic differentiation markers (Fig. 4I), suggesting that miR-34a-5p contributes to the pro-differentiation effect of hAEC-CM on hFOB1.19 cells.

Discussion

Totipotency has previously been suggested to be a prerequisite of ESCs; however, as observed in the present study and former assays (2), hAECs expressed pluripotent stem cell-specific transcription factors and were able to differentiate into all three germ layers. However, it appears that only certain stem cell behaviors are inherited, as hAECs do not express telomerase and are non-tumorigenic upon transplantation (2). The relatively easy isolation method and large yield of hAECs, even from small amnion regions, as observed in the present study, indicate that hAECs may be a promising source of stem cells for regenerative medicine.

In recent years, CM derived from stem cells has been demonstrated to exert beneficial effects, analogous to the direct usage of stem cells (19), and this paracrine effect of stem cells is currently widely accepted as a novel application in tissue engineering. Bioactive molecules in CM have been proven to be effective chemokines for precursor cells in the

host, and are able to exert trophic and immunoregulatory effects on host tissue cells (14,20,21). As precursor cells in bone tissue, OBs make bone regeneration achievable through increases in cell number and activity. In this study, the effects of hAEC-derived CM on the function of the human fetal OB cell line hFOB1.19 were investigated. The results demonstrated that hAEC-CM acts as a robust chemokine for hFOB1.19 cells, and may affect the function of hFOB1.19 cells in a trophic manner.

The paracrine effects of stem cells rely on the presence of a range of bioactive soluble factors, such as growth factors and cytokines (14,22,23), and extracellular vesicles (17,20). Biologically active molecules, such as proteins, mRNA and miRNAs, may be carried inside extracellular vesicles to enable the exchange of genetic material between cells (16,17,24). In our assays, a higher expression of $TGF\beta_1$ and miR-34a-5p was detected in hAECs, suggesting that these factors may contribute to the paracrine effects of hAECs.

As a member of the TGFβ supergene family, $TGF\beta_1$ is most abundantly expressed in the bone, and serves important roles in bone physiology and homeostasis. $TGF\beta_1$ is a multifunctional cytokine that regulates a broad range of biological processes, including the proliferation, migration and differentiation of cells. TGFβ transmits its signals into cells predominantly through the activation of Smad2/3, and the mitogen-activated protein kinase, nuclear factor-κB and phosphoinositide 3 kinase/AKT pathways have been implicated in the functional regulation of $TGF\beta_1$ (25). The effects of $TGF\beta_1$ on OBs may vary greatly depending on the culture conditions, cell types and species of origin (26-28). In the present study, $TGF\beta_1$ was found to stimulate the migration and osteogenic differentiation of hFOB1.19 cells, which is consistent with previous study (29). Furthermore, cell migration and differentiation were downregulated by hAEC-CM depleted of $TGF\beta_1$ by a neutralizing antibody. Collectively, these data indicate that $TGF\beta_1$ contributes to the paracrine effects of hAECs on OBs.

miR-34a-5p was previously demonstrated to promote osteogenic differentiation of human adipose-derived stem cells through the targeting of retinoblastoma-binding protein 2, Notch 1 and cyclin D1 (30), suggesting that an miR-34a-5p-targeted therapy may be a valuable method of promoting bone regeneration. Additionally, miR-34a-5p was previously found to inhibit the cell cycle by targeting cell cycle-related proteins (31,32), including CDK4, CDK6 and cyclin D1; cell proliferation and migration may also be inhibited by the repression of c-Met (32). In the present study, relatively higher expression levels of miR-34a-5p in hFOB1.19 cells were accompanied by significant reductions in cell proliferation and migration. This indicated that miR-34a-5p did not contribute to the positive paracrine effects of hAECs on the proliferation and migration of hFOB1.19 cells.

In our study, the miR-34a-5p levels were found to increase during the differentiation of hFOB1.19 cells, suggesting that miR-34a-5p expression is positively associated with the differentiation of OBs. Furthermore, cell differentiation was significantly enhanced in hFOB1.19 cells transfected with miR-34a-5p mimics, while the pro-differentiation effects of hAEC-CM were downregulated following miR-34a-5p inhibition, thus indicating a stimulatory role of miR-34a-5p in the differentiation of OBs. This study also demonstrated that miR-34a-5p in hAEC-CM was transferred into adjacent hFOB1.19 cells, thus verifying the involvement of miR-34a-5p in the pro-differentiation effect of hAEC-CM on OBs.

In conclusion, hAECs exerted a robust paracrine effect on the function of OBs, and may be a promising cell source for bone regeneration. $TGF\beta_1$ and miR-34a-5p were also identified as potential contributors to the differentiation of OBs. However, various bioactive factors are present in hAEC-CM, and the functions of hAEC-CM are likely a result of the integrated effects of all these factors combined. Therefore, other potential mechanisms require investigation to elucidate the paracrine effects of hAECs in bone regeneration.

Acknowledgements

The present study was supported by the Program for National Basic Research Program of China (grant no. 2012CB518103), the National Natural Science Foundation of China (grant no. 81450017), the Science and Technology Planning Project of Liaoning Province (grant no. 2014305012), the Science and Technology Planning Project of Shenyang (grant no. F14-201-4-00), and the Shenyang Key Laboratory Project (grant no. F15-157-1-00).

References

1. Grayson WL, Bunnell BA, Martin E, Frazier T, Hung BP and Gimble JM: Stromal cells and stem cells in clinical bone regeneration. Nat Rev Endocrinol 11: 140-150, 2015.
2. Miki T, Lehmann T, Cai H, Stolz DB and Strom SC: Stem cell characteristics of amniotic epithelial cells. Stem Cells 23: 1549-1559, 2005.
3. Bilic G, Zeisberger SM, Mallik AS, Zimmermann R and Zisch AH: Comparative characterization of cultured human term amnion epithelial and mesenchymal stromal cells for application in cell therapy. Cell Transplant 17: 955-968, 2008.
4. Magatti M, Caruso M, De Munari S, Vertua E, De D, Manuelpillai U and Parolini O: Human amniotic membrane-derived, esenchymal and epithelial cells exert different effects on monocyte-derived dendritic cell differentiation and function. Cell Transplant 24: 1733-1752, 2015.
5. Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, van Griensven M, Stadler G, Redl H and Gabriel C: Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: A comparison with human mesenchymal stem cells from adipose tissue. Tissue Eng 13: 1173-1183, 2007.
6. Zhou Q, Liu XY, Ruan YX, Wang L, Jiang MM, Wu J and Chen J: Construction of corneal epithelium with human amniotic epithelial cells and repair of limbal deficiency in rabbit models. Hum Cell 28: 22-36, 2015.
7. Vosdoganes P, Wallace EM, Chan ST, Acharya R, Moss TJ and Lim R: Human amnion epithelial cells repair established lung injury. Cell Transplant 22: 1337-1349, 2013.
8. Vosdoganes P, Lim R, Koulaeva E, Chan ST, Acharya R, Moss TJ and Wallace EM: Human amnion epithelial cells modulate hyperoxia-induced neonatal lung injury in mice. Cytotherapy 15: 1021-1029, 2013.
9. Si J, Dai J, Zhang J, Liu S, Gu J, Shi J, Shen SG and Guo L: Comparative investigation of human amniotic epithelial cells and mesenchymal stem cells for application in bone tissue engineering. Stem Cells Int 2015: 565732, 2015.
10. Jiawen S, Jianjun Z, Jiewen D, Dedong Y, Hongbo Y, Jun S, Xudong W, Shen SG and Lihe G: Osteogenic differentiation of human amniotic epithelial cells and its application in alveolar defect restoration. Stem Cells Transl Med 3: 1504-1513, 2014.
11. Dittmer J and Leyh B: Paracrine effects of stem cells in wound healing and cancer progression (Review). Int J Oncol 44: 1789-1798, 2014.
12. Baraniak PR and McDevitt TC: Stem cell paracrine actions and tissue regeneration. Regen Med 5: 121-143, 2010.

13. Yao X, Guo Y, Wang Q, Xu M, Zhang Q, Li T and Lai D: The Paracrine effect of transplanted human amniotic epithelial cells on ovarian function improvement in a mouse model of chemotherapy-induced primary ovarian insufficiency. Stem Cells Int 2016: 4148923, 2016.
14. Ando Y, Matsubara K, Ishikawa J, Fujio M, Shohara R, Hibi H, Ueda M and Yamamoto A: Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. Bone 61: 82-90, 2014.
15. Wang Y, Ma J, Du Y, Miao J and Chen N: Human amnion-derived mesenchymal stem cells protect human bone marrow mesenchymal stem cells against oxidative stress-mediated dysfunction via ERK1/2 MAPK signaling. Mol Cells 39: 186-194, 2016.
16. van der Pol E, Böing AN, Harrison P, Sturk A and Nieuwland R: Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev 64: 676-705, 2012.
17. Desrochers LM, Antonyak MA and Cerione RA: Extracellular vesicles: Satellites of information transfer in cancer and stem cell biology. Dev Cell 37: 301-309, 2016.
18. Davis LA, Dienelt A and zur Nieden NI: Absorption-based assays for the analysis of osteogenic and chondrogenic yield. Methods Mol Biol 690: 255-272, 2011.
19. An JH, Park H, Song JA, Ki KH, Yang JY, Choi HJ, Cho SW, Kim SW, Kim SY, Yoo JJ, *et al*: Transplantation of human umbilical cord blood-derived mesenchymal stem cells or their conditioned medium prevents bone loss in ovariectomized nude mice. Tissue Eng Part A 19: 685-696, 2013.
20. Burke J, Kolhe R, Hunter M, Isales C, Hamrick M and Fulzele S: Stem cell-derived exosomes: A potential alternative therapeutic agent in orthopaedics. Stem Cells Int 2016: 5802529, 2016.
21. Furuta T, Miyaki S, Ishitobi H, Ogura T, Kato Y, Kamei N, Miyado K, Higashi Y and Ochi M: Mesenchymal stem cell-derived exosomes promote fracture healing in a mouse model. Stem Cells Transl Med 5: 1620-1630, 2016.
22. Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi H and Ueda M: Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng Part A 18: 1479-1489, 2012.
23. Meirelles LS, Fontes AM, Covas DT and Caplan AI: Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev 20: 419-427, 2009.
24. Lawson C, Vicencio JM, Yellon DM and Davidson SM: Microvesicles and exosomes: New players in metabolic and cardiovascular disease. J Endocrinol 228: R57-R71, 2016.
25. Javelaud D and Mauviel A: Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: Implications for carcinogenesis. Oncogene 24: 5742-5750, 2005.
26. Kassem M, Kveiborg M and Eriksen EF: Production and action of transforming growth factor-beta in human osteoblast cultures: Dependence on cell differentiation and modulation by calcitriol. Eur J Clin Invest 30: 429-437, 2000.
27. Janssens K, ten Dijke P, Janssens S and Van Hul W: Transforming growth factor-beta1 to the bone. Endocr Rev 26: 743-774, 2005.
28. Ehnert S, Baur J, Schmitt A, Neumaier M, Lucke M, Dooley S, Vester H, Wildemann B, Stöckle U and Nussler AK: TGF-β1 as possible link between loss of bone mineral density and chronic inflammation. PLoS One 5: e14073, 2010.
29. Eichner A, Brock J, Heldin CH and Souchelnytskyi S: Bone morphogenetic protein-7 (OP1) and transforming growth factor-beta1 modulate 1,25$(OH)_2$-vitamin D_3-induced differentiation of human osteoblasts. Exp Cell Res 275: 132-142, 2002.
30. Fan C, Jia L, Zheng Y, Jin C, Liu Y, Liu H and Zhou Y: MiR-34a promotes osteogenic differentiation of human adipose-derived stem cells via the RBP2/NOTCH1/CYCLIN D1 coregulatory network. Stem Cell Reports 7: 236-248, 2016.
31. Komori T: Glucocorticoid signaling and bone biology. Horm Metab Res 48: 755-763, 2016.
32. Yan K, Gao J, Yang T, Ma Q, Qiu X, Fan Q and Ma B: MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both in vitro and in vivo. PLoS One 7: e33778, 2012.