Human amniotic epithelial cells regulate osteoblast differentiation through the secretion of TGFβ₁ and microRNA-34a-5p

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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 β₁ (TGFβ₁) and microRNA 34a-5p (miR-34a-5p) were detected in hAECs. Furthermore, it was demonstrated that TGFβ₁ and miR-34a-5p stimulated the differentiation of hFOB1.19 cells, and that TGFβ₁ promoted cell migration. Moreover, the effects of hAEC-CM were downregulated following the depletion of either TGFβ₁ or miR-34a-5p. These results demonstrated that hAECs promote OB differentiation through the secretion of TGFβ₁ and miR-34a-5p, and that hAECs may be an optimal cell source in bone regenerative medicine.

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 enhanced by surrounding hAECs. The effects of conditioned medium (CM) from hAECs on the function of hFOB1.19 cells was markedly enhanced by surrounding hAECs.

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. 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 washed with cold PBS and then ~1x10⁶ cells were resuspended in complete hAEC medium.

To induce osteogenic differentiation, hAECs were treated with 10 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 ~25-cm² 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 washed with cold PBS and then ~1x10⁶ cells were resuspended in complete hAEC medium.

Cell immortalization. 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 DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin.
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 immunofluorescence 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 cm² 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 CO₂ 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 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 ~0.1 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’-CCAAGGACGTGGAAAATCTTATTGCATGACAGTGAGGGC-3’; osteocalcin (OCN), 5’-GACGAGTTGGCTGACCACAAGGGGAAGAGGAAAGG-3’; osteopontin (OPN), 5’-GATGAATCTGATGAACTGGTCACTGTTGGTAGGCG-3’; runt-related transcription factor 2 (RUNX2), 5’-TAGGCGCATTTCAGGTGCTTGATAGGGG-3’; and β-actin, 5’-AGGAATTCTTATGTTGGCGGCACATAGCACAGCCTGGA-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’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 2, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 3, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’. 

ΔΔCq method. The primer sequences used were as follows: RT primer 1, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 2, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 3, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 4, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’. 

ΔΔCq method. The primer sequences used were as follows: RT primer 1, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 2, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 3, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’; RT primer 4, 5’-GCTGTCAACGATACGCTACGTCATG/GTGGGTAGGGGCAATAGCGG-3’.
ACGAATTGCGTGTCAT-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 TGFβ1 using ELISA. The concentration of TGFβ1 was measured with a Human Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**TGFβ1 assays.** The effects of TGFβ1 on the function of hFOB1.19 cells were evaluated by treating cells with 5 ng/ml recombinant human TGFβ1 protein (PeproTech, Rocky Hill, NJ, USA) in complete medium. Cell proliferation was then assessed as above.

In a separate assay, TGFβ1 contained in the hAEC-CM was neutralized through overnight incubation with 5 µg/ml human TGFβ1 antibody (R&D Systems).

The effects of exogenous TGFβ1 or TGFβ1 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 TGFβ1 in the effects of hAEC-CM.** It was demonstrated by ELISA that the concentration of TGFβ1, was significantly higher in hAEC-CM compared with that in complete (CON) medium (Fig. 3A), indicating that TGFβ1 may contribute to the paracrine effects of hAECs. The effects of TGFβ1 on the function of hFOB1.19 cells were subsequently assessed with recombinant human TGFβ1 protein (5 ng/ml; PeproTech). After hFOB1.19 cells were incubated with TGFβ1 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 TGFβ1 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 TGFβ1 (Fig. 3C). Differences in the activity of ALP were also confirmed with a pNPP assay (Fig. 3D). Collectively, these data indicated that hAEC-CM effectively promoted the function 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,
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.
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
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 TGFβ1 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 TGFβ1 supergene family, TGFβ1 is most abundantly expressed in the bone, and serves important roles in bone physiology and homeostasis. TGFβ1 is a multifunctional cytokine that regulates a broad range of biological processes, including the proliferation, migration and differentiation of cells. TGFβ1 transmits its signals into cells predominantly through the activation of Smad2/3, and the mitogen-activated protein kinase, nuclear factor-xB and phosphoinositide 3 kinase/AKT pathways have been implicated in the functional regulation of TGFβ1 (25). The effects of TGFβ1 on OBs may vary greatly depending on the culture conditions, cell types and species of origin (26-28). In the present study, TGFβ1 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 TGFβ1, by a neutralizing antibody. Collectively, these data indicate that TGFβ1 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. TGFβ1 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.

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