Osteogenic Differentiation of Human Amniotic Epithelial Cells and Its Application in Alveolar Defect Restoration

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INTRODUCTION

Currently, the most widely accepted treatment methods for an alveolar cleft is secondary autogenous bone grafting in mixed dentition [1]. However, donor site defects, potential infection, operative trauma, and even morbidity are among the limitations of autogenous bone graft harvesting. Also, restoration of the alveolar cleft in the pediatric population can be hindered by an inadequate bone graft donor volume. As suggested by the results of recent clinical studies, stem cell-based bone tissue engineering has been recognized as a promising strategy for both aesthetic and functional reconstruction of congenital alveolar clefts [2–5].

Human amniotic epithelial cells (hAECs), developing from the epiblast as early as 8 days after fertilization, have recently been reported to combine qualities from both embryonic and adult stem cells and to retain a remarkable plasticity [6, 7]. hAECs have been shown to possess trilineage differentiation potential and a modulatory influence on the early tissue remodeling process, making these cells a potential source of progenitor cells for clinical restoration of the alveolar defect.
defect using a novel Sprague-Dawley (SD) rat alveolar cleft model. The data reported indicate that hAECs possess proper osteogenic differentiation potential and a localized modulatory influence on the early tissue remodeling process, making these cells a promising stem cell source for congenital alveolar cleft restoration.

**MATERIALS AND METHODS**

**hAEC Isolation and Characterization**

**hAEC Isolation and Culture**

Human amnion membranes were obtained with written and informed consent from healthy mothers undergoing cesarean section. All patients were negative for human immunodeficiency virus-1, hepatitis B, and hepatitis C. The hAECs were isolated and expanded as previously reported [19]. The expansion culture medium (EXP-CM) for hAECs was prepared with Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen, Beijing, China, http://www.invitrogen.com) supplemented with 2 mM L-glutamine (Invitrogen), 10 ng/ml recombinant human epidermal growth factor (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 20% fetal bovine serum (FBS) (Gibco, Invitrogen), and 1% penicillin-streptomycin (Invitrogen).

**Assessment of hAEC Proliferation In Vitro**

hAECs at passages 0, 1 (p1), and 5 were seeded at low density (1 × 10^3 cells per well) into a 96-well plate and cultured for 4 and 2, 4, 6, 8, 10, and 12 days. At each predetermined point, cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Dojindo, Japan, http://www.dojindo.com) according to the manufacturer’s instructions. Triplicate samples were tested in each group at each incubation time.

**Flow Cytometric Analysis**

p1 hAECs were harvested with trypsin/EDTA (Invitrogen) and washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. Approximately 1 × 10^5 cells were incubated with the following phycoerythrin (PE)-upregulated or fluorescein isothiocyanate (FITC)-conjugated anti-human primary antibodies (all purchased from Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.milenyi-biotec.com) in the dark for 10 minutes at 4°C: PE-labeled CD44, FITC-labeled CD45, PE-labeled CD90, FITC-labeled CD34, PE-labeled CD105, FITC-labeled SSEA-4, and FITC-labeled HLA-DR. Nonspecific fluorescence was determined using isotype-matched monoclonal antibody controls. The flow cytometry data were analyzed using Cell Laboratory Quanta SC (Beckman Coulter, Fullerton, CA, http://www.beckmancouler.com) analysis software.

**In Vitro Osteogenic Differentiation and Biochemical Staining for Osteoblasts**

**Osteogenic Induction of hAECs In Vitro**

For in vitro osteoblastic differentiation, p1 hAECs at 70%–80% confluence in test wells were cultured in classic osteogenic medium (DMEM [Invitrogen], supplemented with 10% FBS [Invitrogen], 0.1 mM ascorbic acid [Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com], 10 mM β-glycerophosphate [Sigma-Aldrich], and 10⁻⁵ M dexamethasone [Sigma-Aldrich]). The osteogenic medium was changed every 3 days, and the experiments were terminated at day 21. The cells cultured in EXP-CM were considered an experiment control. Alkaline Phosphatase Staining

After osteogenic induction for 5 and 10 days, the cells in both groups were fixed with 4% paraformaldehyde solution for 10 minutes at room temperature (RT) and washed in PBS. An ALP staining kit (Beyotime Institute of Biotechnology, Shanghai, China) was used for ALP staining according to the manufacturer’s instructions. All samples were rinsed with PBS and photographed using a light microscope (Axio Scope.A1; Carl Zeiss, Jena, Germany, http://www.zeiss.com) with a digital camera (SPOT Flex; SPOT Imaging Solutions, Sterling Heights, MI, http://www.spotimaging.com).

**Alizarin Red S Staining**

After osteogenic induction for 21 days, the cells in both groups were fixed with 4% paraformaldehyde solution for 10 minutes at RT and washed with pure water twice. Next, all samples were placed in 2% alizarin red S (ARS) (pH 4.2; Sigma-Aldrich) solution for 10 minutes, washed with pure water 3 times, air-dried, and photographed.

**Real-Time Polymerase Chain Reaction and Western Blot**

For osteogenic induction for 0, 5, 10, and 14 days, the cells in both groups were harvested. Total RNA was extracted using RNAliso Plus reagent (Takara, Otsu, Japan, http://www.takara.co.jp) and reverse transcribed into cDNA using a PrimeScript real time polymerase chain reaction (PCR) kit (Takara), according to the manufacturer’s instructions. To analyze the expression of specific genes for osteoblastic differentiation, the expression of Runx2, osterix, ALP, collagen I, and osteopontin (OPN) was determined quantitatively on a real-time PCR machine (ABI 7300; Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) using a SYBR Premix Ex Taq kit (Takara), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene for normalization. The expression of E-cadherin, vimentin, Klf4, Sna1, transforming growth factor-β (TGF-β) receptor type 1, and TGF-β1, key genes involved in epithelial-mesenchymal transformation (EMT) of hAECs [20], were also determined quantitatively. Details of the primers are listed in Table 1. The data were analyzed using the comparative C, method and expressed as the fold change.

To verify the protein level of TGF-β1, the cells in both groups were harvested and lysed with a commercial sodium dodecyl sulfate cell lysis buffer (Beyotime Institute of Biotechnology) at 0, 5, 10, and 14 days after osteogenic induction. Protein extracts were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and subjected to Western blot with primary rabbit monoclonal antibody against TGF-β1 (1:1,000; Abcam, Cambridge, U.K., http://www.abcam.com) and primary mouse monoclonal antibody against GAPDH (1:1,000; Abcam). Images of Western blot were taken with an Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE, http://www.licor.com).

**Cytoimmunofluorescence**

To further verify the results of real-time PCR, the cells in both groups at day 10 were fixed with 4% paraformaldehyde solution and subjected to cytoimmunofluorescence. To confirm osteogenic differentiation of hAECs, samples in both groups were incubated with primary rabbit polyclonal anti-human OPN antibodies (1:100; Proteintech Group, Inc., Chicago, IL) and primary rabbit polyclonal anti-human collagen Iα2 (Col Iα2) antibodies (1:100;
Table 1. Primer sequences used for real-time polymerase chain reaction analysis

| Gene     | Forward Primer sequence | Reverse Primer sequence | Accession no. |
|----------|-------------------------|-------------------------|---------------|
| GAPDH    | GCTCTCCGAAGAATCATCC     | TGGTTACCACTCCTCTTG      | NM_002046.3   |
| COL I    | CAGCCGCTCTACCATACGC     | TTTTGTATCAATCAGCTCGTC   | NM_000088.3   |
| ALP      | ACTCCCACTTCTACGGAAAC    | CCGTGACTCTGACTGCTG      | J04948.1      |
| OPN      | CAGAATGCTGCTCTCTGAA     | GTCAAGGAGCTCTGCTG       | NM_001040058  |
| Osterix  | TAGGAGGAAGATACCATG      | TTCTTTTGTGCTCTTG        | BC101549.1    |
| Runx2    | GTCCACTGCTCTACCTTG      | CACACTCCTCTCCCTTCTG     | BC108920.1    |
| E-cadherin | CAAGTGATCCTCCCATCT      | GGAGCGAAACTGCTCTTAAA    | NM_004360.3   |
| Klf4     | ACAAAGAGTTCCTCAGC       | TAGTGCCCTGGTACCTGA      | NM_004235.4   |
| Snai1    | CAGCAGGTGTGACATTAG      | CCAAAACAGGAGCTGGAAT     | NM_005985.3   |
| Vimentin | AAGTGCCCTGTGACATT       | CAGATTTTGGTCCTCTTG      | NM_003380.3   |
| TGF-β1   | ATTCGAGAGTGCTCGGAA      | CACCTTGGCTGAGTTGAC      | NM_004864.2   |
| TGFBR1   | ACAACAGTATCTTACCATCT    | AACTGAGCAACATATAACCCCA  | XM_005252150.1|

Abbreviations: ALP, alkaline phosphatase; COL I, collagen I; OPN, osteopontin; TGF-β1, transforming growth factor-β1; TGFBR1, transforming growth factor β receptor type 1.

Proteintech Group) overnight at 4°C. To confirm EMT of the hAECS, samples in both groups were incubated in primary rabbit polyclonal anti-human E-cadherin (1:100; Proteintech Group) and mouse monoclonal anti-human vimentin (1:100; Proteintech Group) antibodies overnight at 4°C. Next, the samples were washed with PBS and coincubated with Cy3-labeled goat anti-rabbit IgG (1:500; Beyotime Institute of Biotechnology) and FITC-labeled goat anti-mouse IgG (1:500; Beyotime Institute of Biotechnology). At the end of incubation, the samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1,000; Beyotime Institute of Biotechnology). The Cy3, FITC, and DAPI images were taken separately using a fluorescence microscope (DP72; Olympus, Tokyo, Japan, http://www.olympus-global.com) equipped with a digital image capture system (Olympus).

Cell-Scaffold Construct Assembly and Surgical Procedure

The institutional animal research ethics committee approved the animal study protocol. Commercially available β-TCP scaffolds with defined dimensions (length, 4 mm; height, 3 mm; width, 3 mm) were purchased from the Shanghai Ceramic Institute of Chinese Academy of Science. p1 hAECS were harvested and seeded on the scaffolds, as previously described [21, 22]. After hAECS loading for 24 hours, 3 cells per scaffold construct were subjected to scanning electron microscopy. The cell-scaffold constructs were incubated in EXP-CM for 3 days and applied in the animal study. In brief, SD rats, with a mean age of 7 weeks (range 6–8 weeks), weighing 230–250 g, were randomly and equally divided into 2 groups (n = 4 at each point for quantitative micro-computed tomography (CT) analysis and histological analysis): (a) defect grafting with β-TCP scaffolds (CTR group), and (b) defect grafting with hAECS plus β-TCP scaffolds (EXP group). After successful anesthesia with an intraperitoneal injection (0.3 ml per 100 g of body weight) of ketamine hydrochloride (5%), combined with xylazine (2%), unilateral maxillary alveolar defects were created, modified from previously described methods [23, 24] (supplementary online Fig. 1).

Micro-CT Analysis

Samples for high-resolution micro-CT analysis were harvested en bloc at 4 and 8 weeks postoperatively, formalin-fixed, and imaged at a resolution of 20 mm (Skyscan 1172, Skyscan). Regions of interest of 1 × 2 mm were taken through the entire length of the defect. The threshold value for 3-dimensional restoration and quantification was set at 60. Bone formation within the alveolar defect was evaluated using software provided by the manufacturer.

Histological Analysis

Samples after micro-CT analysis were decalcified in 14% EDTA for 3 weeks and paraffin embedded. For morphological study, 5-mm coronal sections were stained with hematoxylin and eosin (HE) and photographed. For semiquantification of bone formation within the defect, blinded histomorphometric analysis using the software Photoshop CS5 (Adobe Systems Inc., San Jose, CA, http://www.adobe.com) was conducted as previously described [21, 25]. In brief, 5 random HE images obtained at ×200 magnification for each sample in both groups were evaluated blindly, and bone formation in each group at each point is expressed as relative mean bone area [26, 27].

Immunohistological Analysis

For immunohistochemistry analysis, the sections were subjected to immunostaining with rabbit monoclonal anti-rat CD68 (1:100; AbD Serotec, Raleigh, NC, http://www.ab-direc.com) and rabbit polyclonal anti-rat vascular endothelial growth factor (VEGF) (1:100, AbD Serotec) using the ABC method (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Next, all the samples were photographed. Semiquantification of immunohistochemistry was performed again using Photoshop CS5 software (Adobe Systems Inc.), as previously described [21, 25–27].

For immunohistofluorescence study, the sections were incubated with rabbit polyclonal anti-rat OCN, rabbit polyclonal anti-human OPN, and mouse monoclonal anti-human antinuclear antibody (ANA) (1:50; MAB1281; Millipore, Billerica, MA, http://www.millipore.com) and visualized with Cy3-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG. Finally, all the samples were counterstained with DAPI and photographed.

Statistical Analysis

All measurements were collected and are expressed as the mean ± SD, unless otherwise noted. Data for these measurements were analyzed using two-way analysis of variance and Student’s t test. A p value < .05 was considered statistically significant. SPSS version 17.0 (IBM Corp., Armonk, NY, http://www-01.ibm.com/software/analytics/spss/) and GraphPad version 5.0 (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com) were used.
used to analyze and demonstrate the statistical significance of the assays. The significance between groups was marked on the graphs.

**RESULTS**

**Isolation and Characterization of hAECs**

A pure population of hAECs was successfully generated with homogeneous cobblestone-like morphology (Fig. 1A, 1B). After 21 days of osteogenic induction, the hAECs showed significant and persistent morphological changes (Fig. 1C, 1D). The proliferation of hAECs at passages 0, 1, and 5 increased significantly with time, and the proliferation of hAECs at passage 1 was significantly greater than at passage 0 and 5 (p < .05) (Fig. 1E). Flow cytometry analysis revealed that p1 hAECs positively expressed CD44, CD90, CD105, and SSEA-4 and did not express CD34, CD45, or HLA-DR. Values represent the percentages of all assessed cells positively stained by the indicated antigens (bottom of each graph). Nonspecific fluorescence was determined as the blank control using isotype-matched monoclonal antibodies (PE blank, FITC blank). (F): Flow cytometry analysis showed that hAECs expressed CD44, CD90, CD105, and SSEA-4 and did not express CD34, CD45, and HLA-DR. Values represent the percentages of all assessed cells positively stained by the indicated antigens (bottom of each graph). Nonspecific fluorescence was determined as the blank control using isotype-matched monoclonal antibodies (PE blank, FITC blank).

**In Vitro Osteogenic Differentiation of hAECs**

ALP and ARS staining confirmed the progressively increased cellular ALP activity and extracellular mineralization after osteogenic induction. In contrast, low cellular ALP activity and no obvious mineral nodules were observed in the control group (Fig. 1G).

At 10 and 14 days after osteogenic induction, the expression of Runx2, osterix, Col I, ALP, and OPN mRNA in hAECs was significantly upregulated compared with their expression in the control.
group (p < .05) (Fig. 2A). The immunolocalization of Col I and OPN in hAECs after osteogenic induction exhibited a more intense fluorescence compared with cells cultured in EXP-CM (Fig. 2B). All the observations were in agreement with the results from real-time PCR analysis.

**EMT Process During Osteogenic Differentiation of hAECs**

Also, the expression of vimentin, Snai1, transforming growth factor-β receptor type 1 (TGF-βR1), and TGF-β1 in hAECs after osteogenic induction exhibited a significantly greater level compared with the control group, although Klf4 and E-cadherin expression was significantly downregulated during the osteogenic differentiation process of hAECs (Fig. 3A). This functional alteration was further confirmed by cytoimmunofluorescence and Western blot study (Fig. 3B, 3C). No fluorescence labeling of E-cadherin was detected in hAECs cultured in osteogenic medium. In contrast, immunofluorescence labeling of vimentin showed significantly more intense fluorescence compared with the control group.

**In Vivo Defect Healing Analysis**

**Micro-CT Analysis**

The three-dimensional micro-CT examination showed significantly increased bone formation in the EXP group compared with the CTR group. The CTR group remained largely void of mineralized tissue, and the EXP group showed much more infilling with mineralized tissue (Fig. 4A). Micro-CT parameter quantification further demonstrated a significantly greater bone volume/tissue volume ratio, bone mineralization density, and trabecular thickness.

Figure 2. Real-time polymerase chain reaction (PCR) and immunofluorescence study for osteogenic differentiation of hAECs in vitro. (A): Real-time PCR assay of osteoblastic marker genes showed significant upregulation of Runx2, Osx, ALP, Col I, and OPN in hAECs at days 5, 10, and 14 after osteogenic induction compared with those in the control group (*, p < .05). (B): Representative images of OPN and Col I immunofluorescence staining at 10 days of osteogenic differentiation in osteogenic and control groups under epifluorescence microscope. Image magnification, ×200. Scale bar = 50 μm. Abbreviations: ALP, alkaline phosphatase; Col I, collagen I; CTR, control (defect grafting with β-tricalcium phosphate scaffolds); DAPI, 4′6-diamidino-2-phenylindole; hAECs, human amniotic epithelial cells; OPN, osteopontin; Osx, osterix.
in the EXP group than in the CTR group at both 4 and 8 weeks postoperatively (Fig. 4B).

Histological Analysis
HE staining in both groups showed signs of progressive new bone deposition. Defects in the EXP group showed more new bone formation than in the CTR group at both 4 and 8 weeks postoperatively (Fig. 4C). More trabecular bone tissue lined by a continuous layer of osteoblasts was clearly observed at both postoperative points in the EXP group, and the new bone formation in the CTR group was much more limited. Histomorphometric quantification of relative new bone area further supported the progressive bone deposition after surgery in both groups (Fig. 4F).

Figure 3. Real-time polymerase chain reaction (PCR), Western blot, and immunofluorescence study for epithelial-mesenchymal transformation (EMT) process during osteogenic differentiation of hAECs in vitro. (A): Real-time PCR assay of EMT marker genes showed significant upregulation of vimentin, Snai1, TGF-βR1, and TGF-β1 and downregulation of Klf4 and E-cadherin in hAECs at days 5, 10, and 14 after osteogenic induction compared with those in the control group (\( * \), \( p < .05 \)). (B): Representative images of E-cadherin and vimentin immunofluorescence staining at 10 days of osteogenic differentiation in osteogenic and control groups under epifluorescence microscope. Image magnification, \( \times 200 \). Scale bar = 50 μm. (C): Qualitative and semiquantitative Western blot analysis of TGF-β1 expression in hAECs at days 5, 10, and 14 after osteogenic induction compared with those in the control group (\( * \), \( p < .05 \)). Abbreviations: CTR, control (defect grafting with β-tricalcium phosphate scaffolds); DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hAECs, human amniotic epithelial cells; TGF-β1, transforming growth factor-β1; TGFBR1, transforming growth factor-β receptor type 1.
Figure 4. In vivo healing process in alveolar defect at 4 and 8 weeks postoperatively. (A): Representative three-dimensional micro-computed tomography (CT) reconstruction images of hAECs+β-TCP scaffold (EXP) and β-TCP scaffold (CTR) at 4 and 8 weeks postoperatively. Scale bar = 1 mm. (B): Micro-CT parameters acquired among β-TCP scaffold in vitro, hAECs+β-TCP scaffold in vivo, and β-TCP scaffold alone in vivo at 4 and 8 weeks postoperatively (\(p < .05\)). (C): Hematoxylin and eosin staining of the rat alveolar defect at 4 and 8 weeks postoperatively revealed more active new bone formation in the EXP group than in the CTR group (\(\times 50\) and \(\times 200\) magnification). Alveolar defect treated with hAECs+β-TCP scaffold exhibited a more mature lamellae-bone formation at 8 weeks postoperatively. Scale bar = 200 μm. (D): ANA-positive cells, visible as green fluorescence in the nuclei, were observed within the newly deposited OCN, and OPN-positive bone tissue, visible as red fluorescence, in the EXP group at 4 weeks postoperatively, indicating a mature osteoblastic function of these hAEC-derived cells. Scale bar = 50 μm. (E): Representative images of immunohistochemical staining of sections with anti-VEGF antibody and anti-CD68 antibody in hAECs+β-TCP scaffold (EXP) and β-TCP scaffold (CTR) at 4 and 8 weeks postoperatively. Scale bar = 200 μm. (F): The histomorphometric quantification of the relative new bone area, VEGF-positive area, and CD68-positive area showed that more bone tissue regeneration was observed in the EXP group than in the CTR group at 4 and 8 weeks postoperatively. The positive signal of VEGF and CD68 in the EXP group was much weaker at 4 weeks postoperatively and became more intense at 8 weeks postoperatively compared with the CTR group (\(\times 50\), \(p < .05\)). Abbreviations: ANA, anti-nuclear antibody; BV/TV, bone volume/tissue volume ratio; BMD, bone mineralization density; hAECs, human amniotic epithelial cells; OPN, osteopontin; post op, postoperatively; SMI, structure model index; Tb.Th., trabecular thickness; Tb.N., trabecular number; Tb.Sp., trabecular separation; β-TCP, β-tricalcium phosphate; VEGF, vascular endothelial growth factor.
To further study the involvement of hAECs in new bone formation in vivo, immunohistofluorescence staining for human nuclei revealed that some ANA-positive cells, visible as green fluorescence in the nuclei, existed within the newly deposited bone tissue at 4 weeks postoperatively. OCN and OPN immunohistofluorescence staining, visible as red fluorescence, was clearly detected within the bone matrix around these implanted hAECs, indicating a direct participation of the transplanted hAECs in the restoration of the alveolar defect in SD rats (Fig. 4D).

In the evaluation of the tissue remodeling and scaffold absorption process, immunohistochemical staining for the pan-macrophage marker CD68 was also performed. CD68-positive macrophages were mostly found in the capsule at the periphery of the scaffold remnants (Fig. 4E). Histomorphometric quantification of positive staining intensity for CD68 demonstrated more pronounced macrophage activity in the CTR group at 4 weeks postoperatively, and samples in the EXP group showed a significantly delayed macrophage response (Fig. 4F).

In the evaluation of angiogenesis, the CTR group exhibited relatively higher levels of VEGF staining and a more widespread distribution of VEGF at 4 weeks postoperatively. The EXP group demonstrated moderate levels of VEGF staining. A significantly increased level of VEGF staining was observed in the EXP group at 8 weeks postoperatively, and VEGF staining in the CTR group was much milder (Fig. 4E, 4F).

### DISCUSSION

In the present study, we assessed the detailed osteogenic differentiation process of hAECs in vitro and used the cells and β-TCP scaffolds in the restoration of SD rat alveolar defects to illustrate their osteogenic potential in vivo. Our results have strongly validated the in vitro osteogenic capacity of hAECs and indicated that an EMT process might be involved in the osteogenic differentiation of hAECs through upregulation of TGF-β1. Our data also demonstrated that in vivo implantation of hAECs loaded on the β-TCP scaffold, not only improved bone regeneration by direct participation in bone repair of the alveolar defect, but also modulated the localized immune response to the scaffolds.

In consideration of our previous study showing that osteogenic differentiation of hAECs could be significantly reduced after in vitro cultivation for 2 passages, the hAECs used in the present study were harvested at passage 1 with a previously described cell surface phenotype defined as positive expression of CD44, CD90, CD105, and SSEA-4 and negative expression of HLA-DR, CD34, and major histocompatibility complex class I and II expression, supporting the use of AECs xenogeneically [14, 15, 31–34]. Intravenous delivery of fresh, unfractionated hAECs into newborn rats, or their interperitoneal delivery into newborn swine, led to microchimerism in multiple organs and tissues [31]. In agreement with previous studies on the immunoprivileged properties of hAECs [13, 15, 16], our immunohistofluorescence staining findings confirmed that some transplanted hAECs survived and directly participated in the process of new bone deposition at 4 weeks postoperatively.

Moreover, hAECs were widely accepted to be not only non-immunogenic or hypoimmunogenic but also immunomodulatory and long-term antifibrotic in vitro and in vivo [14–16, 33, 34]. Because scaffolds can induce variable immune responses in vivo, which are generally characterized by the infiltration of macrophages and lymphocytes, macrophages and inflammatory cells play an important role in the degradation of the implanted scaffold and the early bone healing process [35, 36]. Ideally, this particular scaffold degradation would be appropriately consistent with the remodeling process of the new bone tissue, such that

significantly upregulated after 14 days of osteoblastic culture without the addition of any bone morphogenetic proteins. hAECs in osteoblastic culture underwent dramatic morphological changes, acquiring a mesenchymal shape. Cultured hAECs at high passages have been widely reported to lose their epithelial morphology and convert to mesenchymal stromal-like cells. Very recently, Alcaraz et al. demonstrated that long-term cultured hAECs underwent bona fide EMT through an autocrine TGF-β1-dependent process characterized by increased expression of vimentin and Snai1 and decreased expression of E-cadherin, Mta3, and Klf4 [20, 29]. Our results, in contrast, have demonstrated that in vitro osteoblastic culture significantly upregulated the expression of TGF-β1 in hAECs, which might further accelerate this EMT process of hAECs.

To further investigate the osteogenic potential of hAECs in vivo, a novel SD rat alveolar defect model was used in our study. Although several animal models, such as the canine periodontal bone defects model and rat calvarial defect model, have been widely used and accepted as reproducible models for craniofacial bone regeneration, these animal models might not be specific and suitable for applied research of the alveolar cleft defect. With the high incidence of the alveolar cleft, our rat model might serve as a reproducible and cost-effective small animal model suitable for alveolar cleft restoration. According to our own experience and recent reports, hAECs were preferably loaded on the scaffold and cultured for a shorter period in vitro before implantation [2, 12, 30]. The β-TCP scaffold with proven osteocompatibility and low osteoinducitvity was used for easier investigation of the in vivo osteogenic differentiation of hAECs. Our results have demonstrated that hAECs can easily adhere to, and proliferate on, the scaffold and significantly improved bone tissue regeneration after in vivo transplantation. Both micro-CT and histological analysis demonstrated that the process of bone regeneration was accelerated and enhanced in the EXP group at both 4 and 8 weeks postoperatively compared with that in the CTR group. A significantly greater amount of bone tissue was regenerated in the EXP group, especially at 8 weeks postoperatively. In contrast, only a minimal amount of new bone was observed at the inner site of the defect in the CTR group.

Important for transplantation approaches, hAECs have been evaluated in a variety of animal models of human disease without evidence of xenogeneic reactions and have exhibited minimal major histocompatibility complex class I and II expression, supporting the use of AECs xenogeneically [14, 15, 31–34]. Intravenous delivery of fresh, unfractionated hAECs into newborn rats, or their interperitoneal delivery into newborn swine, led to microchimerism in multiple organs and tissues [31]. In agreement with previous studies on the immunoprivileged properties of hAECs [13, 15, 16], our immunohistofluorescence staining findings confirmed that some transplanted hAECs survived and directly participated in the process of new bone deposition at 4 weeks postoperatively.

The in vitro osteogenic differentiation of hAECs in our study was strongly confirmed by the upregulation of specific osteogenic genes using both real-time PCR assay and immunofluorescence staining. Runx2, osteix, Col I, ALP, and OPN in hAECs were
the defect would be totally regenerated by the time the whole scaffold has degraded. As demonstrated in our present work by immunohistochemical staining for the pan-macrophage marker CD68, host immunological responses were observed in both groups at the periphery of the scaffolds. However, the inflammation and macrophage responses in the EXP group were much milder than those in the CTR group at 4 weeks postoperatively. In addition, more scaffold remnants were detected in the EXP group at 4 weeks postoperatively using micro-CT analysis. Thus, the transplanted hAECs might have exerted a positive influence on the early tissue remodeling process in the defect region and prevented the widely described overfast degradation rate of the β-TCP scaffold. Not consistent with the results of a recent study by Barboni et al. [12], VEGF expression in the CTR group was significantly greater than that in the EXP group at 4 and 8 weeks postoperatively, especially at the periphery of the scaffolds. This different angiogenic behavior could be interpreted as a consequence of the greater inflammatory reaction in the control group.

CONCLUSION

Our present work has demonstrated the detailed osteogenic differentiation process of hAECs. An EMT process might be involved in this process through upregulation of TGF-β1. Moreover, our study has provided the first evidence that hAECs exhibit direct involvement in new bone regeneration and a localized modulatory influence on the early tissue remodeling process, indirectly contributing to osteogenesis in the alveolar defect. Altogether, these results imply the potential clinical indication of hAECs as an alternative stem cell-based approach in the restoration of the alveolar cleft. However, we must admit that the exact role of hAECs in the xenogeneic transplant model are far from clear. Further understanding of their mechanism in self-renewal, differentiation, and immunogenicity is needed. Moreover, a systemic comparison of their regenerative potential and establishment of the optimal cell dose, passage number, transplantation window for bone regeneration, and many other parameters must be verified before the feasibility of hAEC use can be determined.

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AUTHOR CONTRIBUTIONS

S. Jaiwen: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Z.J.: conception and design, provision of study material, manuscript writing; D.J.: collection and/or assembly of data, administrative support; Y.D.: data analysis and interpretation; Y.H.: administrative support, conception and design; S. Jun: human platelet lysate preparation, production, and cell culture; W.X.: administrative support, conception and design; S.G.F.S. and G.L.: financial support, conception and design, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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