·Basic Research·

In vitro reconstruction and characterization of tissue-engineered human corneal epithelium with seeder cells from an untransfected human corneal epithelial cell line

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Abstract

• AIM: To demonstrate the morphology and structure of *in vitro* reconstructed tissue-engineered human corneal epithelium (TE-HCEP) with seeder cells from an untransfected HCEP cell line.

• METHODS: The TE-HCEPs were reconstructed *in vitro* with seeder cells from an untransfected HCEP cell line, and scaffold carriers of denuded amniotic membrane (dAM) in air-liquid interface culture for 3, 5, 7 and 9 days, respectively. The specimens were examined with hematoxylin-eosin (HE) staining of paraffin-section, immunocytochemical staining, scanning and transmission electron microscopy.

• RESULTS: During *in vitro* reconstruction of TE-HCEP, HCEP cells formed a 3-4, 6-7 and 8-10 layers of an HCEP-like structure on dAMs in air-liquid interface culture for 3, 5 and 7 days, respectively. But the cells deceased to 5-6 layers and the structure of straified epithelium became loose at day 9. And the cells maintained positive expression of marker proteins (keratin 3 and keratin 12), cell-junction proteins (zonula occludens-1, E-cadherin, connexin 43 and integrin β 1) and membrane transport protein of Na^+-K^+ ATPase. The HCEP cells in TE-HCEP were rich in microvilli on apical surface and established numerous cell-cell and cell-dAM junctions at day 5.

• CONCLUSION: The morphology and structure of the reconstructed TE-HCEP were similar to those of HCEP *in vivo*. The HCEP cells in the reconstructed TE-HCEP maintained the properties of HCEP cells, including abilities of forming intercellular and cell-extracellular matrix junctions and abilities of performing membrane transportation. The untransfected HCEP cells and dAMs could promisingly be used in reconstruction HCEP equivalent for clinical corneal epithelium transplantation.

• KEYWORDS: tissue-engineered human corneal epithelium; *in vitro* reconstruction; untransfected human corneal epithelial cell; denuded amniotic membrane

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INTRODUCTION

Human corneal epithelium (HCEP), with integrity and no blood vessels, is crucial for maintaining corneal transparency and normal physiological function [1]. The maintenance of a healthy corneal epithelium is provided by a unique subpopulation of limbal epithelial stem cells (LESCs) [2,3]. Extensive microbial infection, chemical or thermal burns, contact lens wear, multiple surgeries, Stevens-Johnson syndrome, and so on often cause HCEP cell and/or LESC deficiency including conjunctivalization, epithelial defects, chronic inflammation, scarring and ulcerations which often result in edema and turbidity of cornea [4,5].

Amniotic membrane (AM), with low or no immunogenicity, which is frequently used as a graft for ocular surface reconstruction and successful re-epithelialization, has been successfully used in ophthalmology [6,7]. LESCs [8,9-11] and AMs [6,7,12] have been clinically applied in treatment of LESC

deficiency. However, autologous LESC transplantation is highly restricted to patients with unilateral LESC deficiency [13,14]. An alternative approach by transplantation of cultivated allogeneic LESCs is also limited because of their short life span, rapid differentiation and limited availability of donor corneal tissues [15,16]. At present, tissue-engineered HCEP (TE-HCEP) is now considered as an ideal HCEP equivalent for the therapy of HCEP cell deficiencies [17,18]. Since an untransfected HCEP cell line (utHCEPC01), which is highly biocompatible with denuded AM (dAM), has been successfully established in our laboratory [16], reconstruction of TE-HCEPs can be performed *in vitro* with untransfected HCEP cells and dAMs. To obtain an ideal HCEP equivalent and lay foundation for its future clinical application, *in vitro* reconstruction and characterization of TE-HCEP were performed by using HCEP cells from utHCEPC01 cell line as seeder cells and dAMs as scarffold carriers in this study.

MATERIALS AND METHODS

Materials Untransfected HCEP cells at passage 80 from the utHCEPC01 cell line, established previously in our laboratory with approved corneas donated from a 26 years old woman, was cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (1:1,v:v)(DMEM/F12) medium containing 20% fetal bovine serum (FBS)(HyClone, Logan, Utah) (Invitrogen, Carlsbad, CA)(pH 7.2) at 37 ℃ in a 5% CO_2 incubator as described previously [16]. Fresh AMs were obtained from Shandong Eye Institute of Shandong Medical Academy, Qingdao, China, and denuded by trypsinization with 0.02% EDTA-0.25% trypsin (Sigma-Aldrich, St. Louis, MO)(1:1, v:v) solution to obtain dAMs according to Fan *et al*[19].

Methods

***In vitro* reconstruction of TE-HCEP** HCEP cells at logarithmic phase were collected using 0.25% trypsin (Sigma-Aldrich) as described previously [16]. After cell number counted with a Casy Model DT cell counter (Schärfe System, Reutlingen, German), the density of cell suspension was adjusted to 1.0×10^7/mL with 15% FBS-DMEM/F12 medium (pH 7.2). To each of a dAM-paved culture insert in a 24-well plate, 500μL cell suspension was plated and cultured at the same conditions as described above for 12 hours. Then the culture inserts were transferred into a 6-well plate wells containing 0.8mL 10% FBS-DMEM/F12 medium (pH 7.2) and cultured in air-liquid interface culture. The medium was refreshed daily, and the morphology and growth status of the HCEP cells were monitored with an Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan).

Histological characterization of TE-HCEP After air-liquid interface cultured for 3, 5, 7 and 9 days, the reconstructed TE-HCEP were harvested respectively. The histological property of the reconstructed TE-HCEP was examined with paraffin section and hematoxylin-eosin (HE) staining. The surface morphology of the reconstructed TE-HCEP was examined with a JSM2840 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). And the multilayer structure and attachment status to dAM of the reconstructed TE-HCEP were examined with a H700 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan).

Immunocytochemical characterization of TE-HCEP The expression patterns of marker proteins (keratin 3, keratin 12) and function proteins including cell junction proteins (zonula occludens-1, E-cadherin, connexin 43, integrin β1) and membrane transport protein (Na^+-K^+ ATPase) of the reconstructed TE-HCEP were examined with freeze sections as described previously [16]. Each of freeze sections was incubated with monoclonal antibodies of mouse anti-human keratin 3, keratin 12, zonula occludens-1, E-cadherin, connexin 43, integrin β1 and Na^+-K^+ ATPase (Santa Cruz Biotechnology) at 4℃ overnight, respectively, according to manufacturer's instructions. After incubated with fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Biosynthesis Biotechnology, Beijing, China) at 37℃ for 1 hour, the wells were analyzed with a Ti-S inverted fluorescent microscope (Nikon). Omission of primary antibodies was used as controls.

Statistical Analysis Data were expressed as mean±SD in triplicates and tested for statistical significance with ANOVA single factor.

RESULTS

***In vitro* Reconstruction of TE-HCEP** The HCEP cells grew well on dAM in cobblestone morphology during air-liquid interface culture (Figure 1). And the cells grew into multilayer and the boundary of cells became unclear with time. The reconstructed TE-HCEPs were highly transparent after air-liquid interface cultured for 5 days (Figure 2).

Histological Characterization of TE-HCEP After air-liquid interface culture for 3 days, the HCEP cells formed a 3-4 layer epithelium-like structure (Figure 3A), and 6-7 and 8-10 layer epithelium-like structures was formed at day 5 and day 7, respectively (Figures 3B,3C). But the number of cells began to decrease and the structure of only a 5-6 layer epithelium became loose and unstratified at day 9 (Figure 3D). The HCEP cells differentiated into flattened epidermal cells on its apical surface and cobblestone epithelial cells inside the multilayer epithelium. Under SEM and TEM, the HCEP cells were rich in microvilli on apical surface(Figures 4A, 4B), and constructed numerous intercellular cell junctions including desmosomes (arrowhead) and cell-dAM hemidesmosomes (arrow) at day 5 (Figures 4C, 4D). All these indicate that the reconstructed TE-HCEP has almost

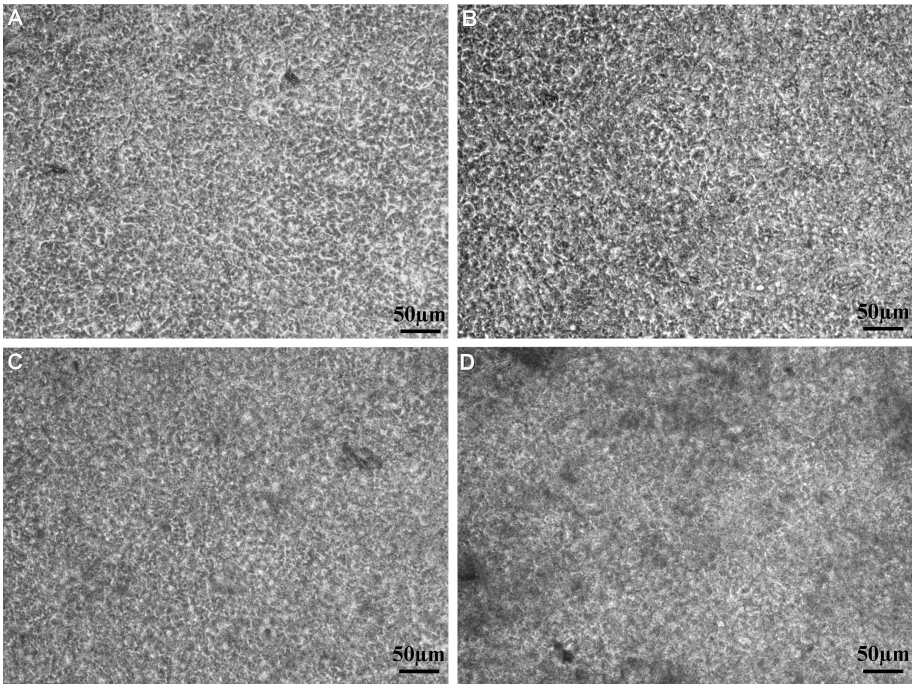

Figure 1 ***In vitro*** **reconstruction of TE-HCEP from untransfected Passage 60 HCEP cells and dAMs in air-liquid interface culture** A: Day 3; B: Day 5; C: Day 7; D: Day 9.

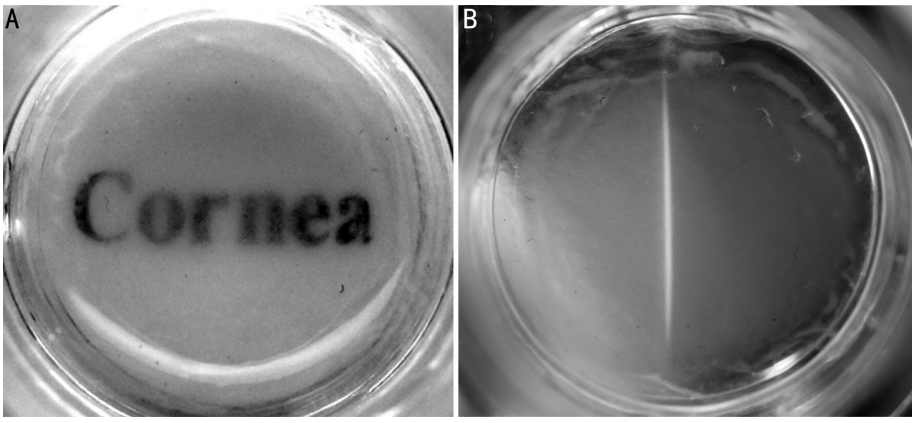

Figure 2 The transparency status of reconstructed TE-HCEP from untransfected Passage 60 HCEP cells and dAMs at day 5 A: Macroscopic view; B: Slit-lamp microscopic view.

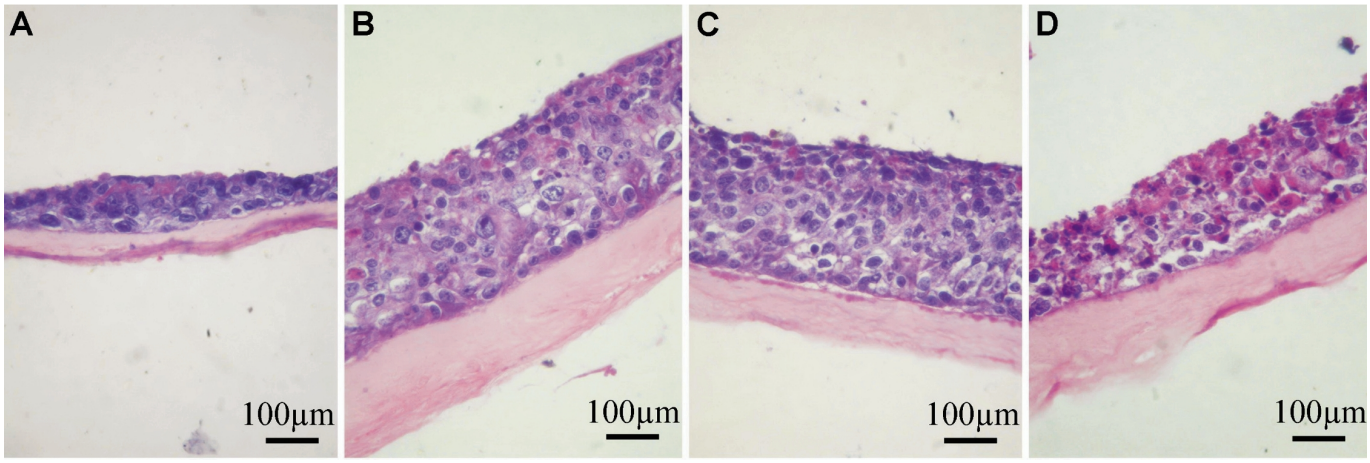

Figure 3 HE staining of reconstructed TE-HCEP from untransfected HCEP cells and dAMs in air-liquid interface culture A: Day 3; B: Day 5; C: Day 7; D: Day 9.

the same morphology and histological structure as that of innate HCEP.

Immunocytochemical Characterization of Marker Protein Expression in TE-HCEP HCEP cells possessed positive expression of marker proteins including keratin 3 and keratin 12) in air-liquid interface culture on dAM at day

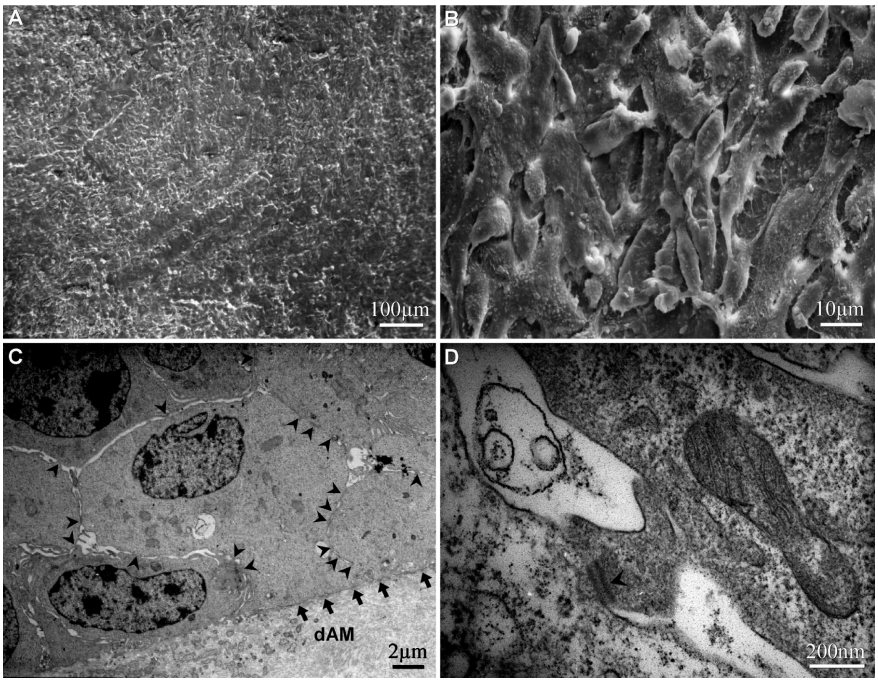

Figure 4 Electron microscopic images of reconstructed TE-HCEP from untransfected HCEP cells and dAMs in air-liquid interface culture at day 5 A, B: SEM; C, D: TEM.

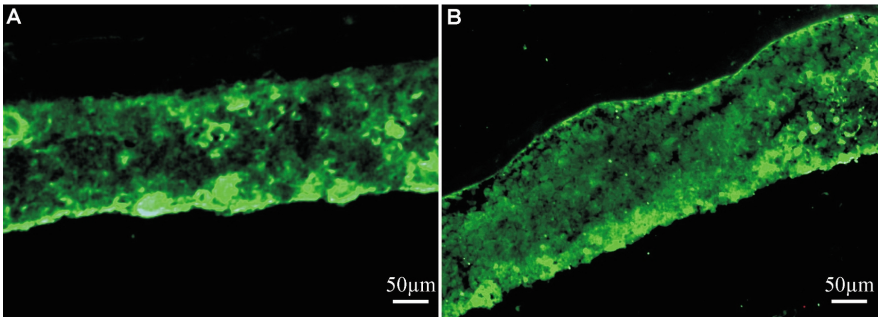

Figure 5 Expression pattern of marker proteins of reconstructed TE-HCEP from untransfected HCEP cells and dAMs in air-liquid interface culture at day 5 A: Keratin 3; B: Keratin 12.

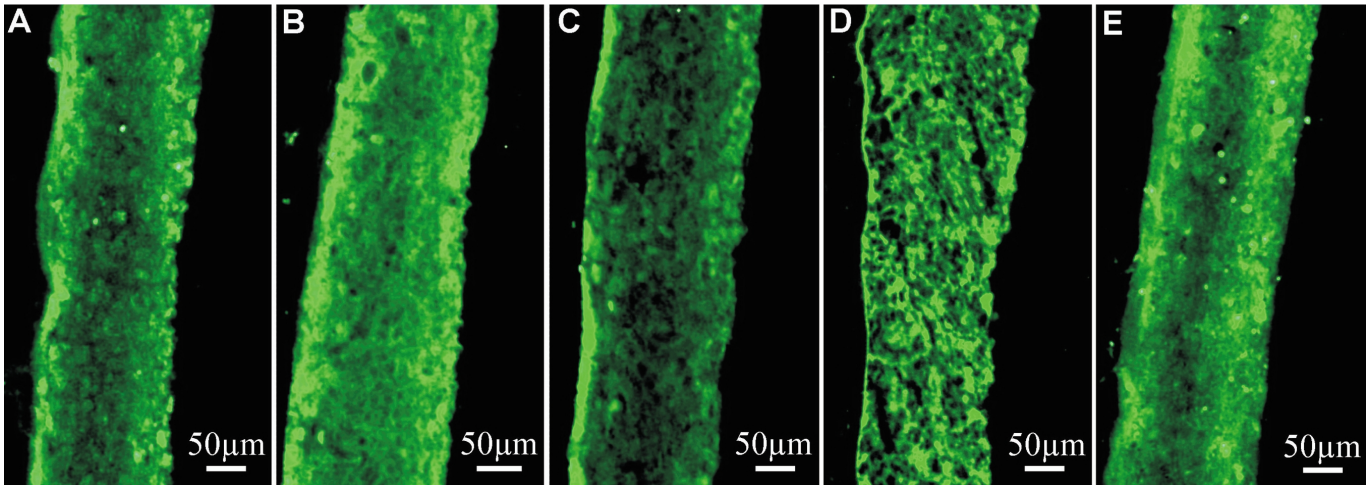

Figure 6 Expression pattern of function proteins of reconstructed TE-HCEP from untransfected HCEP cells and dAMs in air-liquid interface culture at day 5 A: Zonula occludens-1; B: E-cadherin; C: Connexin 43; D: Integrin β1; E: Na^+-K^+ ATPase.

5 (Figure 5), indicating that they preserved the properties of HCEP cells.

Immunocytochemical Characterization of Function Protein Expression in TE-HCEP HCEP cells in reconstructed TE-HCEP expressed positively different cell-junction proteins (zonula occludens-1, E-cadherin, connexin 43, integrin β1) and membrane transport protein (Na^+-K^+ ATPase) in air-liquid interface culture on dAM at day 5 (Figure 6), indicating that they reserved the abilities of forming cell-cell and cell-extracellular matrix (ECM) junctions and abilities of membrane transportation.

DISCUSSION

Due to the relative deficit of LESC donor and difficulty in LESCs culturing, untransfected HCEP cell lines can be used as cell banks of normal HCEP cells. The cell banks can provide a stable source of HCEP cells for TE-HCEP reconstruction and new therapies for HCEP diseases and damages. Since an untransfected HCEP cell line was successfully established in our laboratory [16], *in vitro* reconstruction and characterization of TE-HCEP was performed in this study.

After air-liquid interface cultured on dAM for more than 3 days, untransfected HCEP cells from the previously established untransfected HCEP cell line formed a multilayer layer epithelium-like structure with flattened cells on its apical surface and cobblestone cells underneath. And a 6-7 layer and 8-10 layer epithelium-like structure with a continuous layer of flattened apical cells was reconstructed at day 5 and day 7, respectively. The apical cells were flattened and rich in microvilli, which was similar in characteristics to those of squamous cells from HCEP both *in vivo* and *in vitro* [15,16,18]. After reconstructed for 5 days, the cells of TE-HCEP established numerous cell junctions including desmosomes between HCEP cells and hemidesmosomes between cells and dAM which can be visualized under TEM, and the ultrastructure of the HCEP cells was similar to that of HCEP cells *in vivo* [20-22]. All these indicate that the reconstructed TE-HCEP with integrity has almost the same morphology and structure as that of innate HCEP.

Besides, the cells of TE-HCEP maintained positive expression of marker proteins (keratin 3, keratin 12) and function proteins including cell junction proteins (zonula occludens-1, E-cadherin, connexin 43, integrin β1) and the membrane transport protein of Na^+-K^+ ATPase, indicating that cells reserved the properties of normal HCEP cells *in vivo* and abilities of forming cell-cell and cell-ECM junctions and abilities of membrane transportation. Combined with the ultrastructure of HCEP cells, it can be concluded that the ultrastructure, expression of cell-junction protein and membrane transport protein of reconstructed TE-HCEP is same as those of innate HCEP cells, which is better than that reported by Ban and his colleagues [23].

In conclusion, a novel TE-HCEP, with normal morphology and structure, has been successfully reconstructed *in vitro* in this study. It consists of 6-7 and 8-10 layers of HCEP cells, with positive expression of marker proteins and functional proteins, in air-liquid interface culture for 5 and 7 days, respectively. The reconstructed TE-HCEP, as an HCEP equivalent, provides a promising method in regenerative medicine for corneal epithelium reconstruction and corneal epithelial disorder therapy. TE-HCEP transplantation in LESC deficiency rabbit models is ongoing.

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