·Basic Research·

Establishment of an untransfected human corneal stromal cell line and its biocompatibility to acellular porcine corneal stroma

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Abstract

• AIM: To establish an untransfected human corneal stromal (HCS) cell line and characterize its biocompatibility to acellular porcine corneal stroma (aPCS).

• METHODS: Primary culture was initiated with a pure population of HCS cells in DMEM/F12 media (pH 7.2) containing 20% fetal bovine serum and various necessary growth factors. The established cell line was characterized by growth property, chromosome analysis, tumorigenicity assay, expression of marker proteins and functional proteins. Furthermore, the biocompatibility of HCS cells with aPCS was examined through histological and immunocytochemistry analyses and with light, electron microscopies.

• RESULTS: HCS cells proliferated to confluence 2 weeks later in primary culture and have been subcultured to passage 140 so far. A continuous untransfected HCS cell line with a population doubling time of 41.44 hours at passage 80 has been determined. Results of chromosome analysis, morphology, combined with the results of expression of marker protein and functional proteins suggested that the cells retained HCS cell properties. Furthermore, HCS cells have no tumorigenicity, and with excellent biocompatibility to aPCS.

• CONCLUSION: An untransfected and non-tumorigenic HCS cell line has been established, and the cells maintained positive expression of marker proteins and functional proteins. The cell line, with excellent biocompatibility to aPCS, might be used for *in vitro* reconstruction of tissue-engineered HCS.

• KEYWORDS: human corneal stromal cells; cell line; untransfected; biocompatibility; acellular porcine corneal stroma

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INTRODUCTION

The cornea is the transparent fiber membrane in the forefront of the eye ball, it has a variety of important functions such as defense and refractive. And it provides 70% of the refractive power of the eye [1]. It is comprised of three layers: an outer stratified epithelium, a collagen-rich middle stromal layer and an inner single-cell layered endothelium [2]. The corneal stromal cells (keratocytes) are neural crest derived mesenchymal cells that situated between the collagen lamellae in the stroma, which are quiescent cells with a typical dendritic morphology [3]. These cells are sparsely arranged in the stroma, they form a three-dimensional network of cells interconnected by gap-junctions [4]. The corneal stromal cells can produce fibrillar collagens and crystallins. The crystallins help fibrillar collagens to form into a highly organized extracellular matrix (ECM) that collagen fibrils have an uniform diameter and interfibrillar spacing [5]. And the crystallins are known to contribute to the transparent nature of the cornea [1,6,7].

Upon injury, the corneal stromal cells undergo apoptosis, while peripheral corneal stromal cells lose their quiescence and transition into repair phenotypes. These repair phenotypes can become fibroblastic and migrate to the site of injury and become myofibroblasts at last. The myofibroblasts can induce fibrotic scar formation which is detrimental to the transparent cornea [8-10]. The established *in vitro* cell lines from normal corneal stroma would promote the study of growth, differentiation and injury response of

the cornea [11]. Furthermore, corneal tissue engineering also require better characterization of corneal stromal cells and the ability to proliferate in culture without loss of their *in situ* properties[1]. Numerous methods for the primary culture and preservation of the corneal stromal cells have been presented [12-17]. Some have even established immortalized, permanent corneal stromal cell lines by transfection with oncogenes [11,18,19]. However, these transfected cell lines are not suitable for tissue-engineered human corneal stroma (TE-HCS) reconstruction due to their abnormal phenotypes and latent risk of tumorigenicity. The primary or transfected human corneal stromal (HCS) cells have defects which limit the application of tissue engineering corneal stroma either. Thus a long term culturing of untransfected HCS cell line is essential for both academic and clinic purpose. To ensure the features of untransfected HCS cell line, the human corneal stromal cell line is examined by using growth characteristics, chromosome morphological observation, immunocytochemistry analysis, tumorigenicity test and biocompatibility to acellular porcine corneal stroma (aPCS).

MATERIALS AND METHODS

Materials Corneas of a woman (26 years old, died from cerebral hemorrhage) were obtained from the Affiliated Hospital of Medical College, Qingdao University, Qingdao, China with a permission from her next of kin. The usage of the corneas as the source of HCS cells for *in vitro* culture was approved by the Medical Ethics Committee of the hospital and the privacy of the patient was protected in compliance with the Declaration of Helsinki. SPF BalB/c nude mice (male, 18-22g in body weight) used in these experiments were purchased from Slaccas Experimental Animal Co., Ltd. (Shanghai, China). All animals were treated in accordance with the ARVO Statement for usage in tumorigenesis assay, and were approved by the Clinical Research Ethics Committee of New Drug Evaluation Center, Shandong University, Jinan, China. Adult porcine eyes were obtained from a local slaughterhouse (Qindao Kanglilai Food Co., Ltd, Qingdao, China) within 3 hours post mortem, and subjected to corneal decellularization procedure within 1 hour of acquisition.

Methods

***In vitro* culture of HCS cells** After Bowman's membrane and Descemet's membrane were torn off, the stromal samples were cut into small pieces, then placed onto a 35 mm culture dish and immerged with 0.5mL of 0.25% Collagenase (Sigma-Aldrich, St. Louis, MO) for 2 minutes. Subsequently, the tissue pieces were rinsed with Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12, 1:1) medium (pH 7.2) (Invitrogen, Carlsbad, CA) and then attached directly to a 0.01% gelatin (Sigma-Aldrich)-coated wells of a 24-well culture plate and cultured in 1mL of DMEM/F12 medium containing 10% fetal bovine serum (FBS) at 37℃ with 5% CO_2. In about 12 hours, the medium was replaced with 20% FBS-DMEM/F12 medium (pH 7.2) supplemented with 40ng/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich), 20ng/mL epidermal growth factor (EGF) (Sigma-Aldrich), 0.8mg/mL chondroitin sulfate(Sigma-Aldrich),50μg/mL carboxymethyl-chitosan (AK Scientific, Mountain, CA) and 100μg/mL collagen IV (Sigma-Aldrich). The primary culture was carried out at 37℃ with 5% CO_2 by replenishing half of the medium twice a week. When the cells had covered 90% of the area of the flask, HCS cells were collected by trypsinization and subcultured at a ratio of 1:2 as described previously [20,21]. From passage 20, the HCS cells were subcultured in 10% FBS-containing DMEM/F12 medium (pH 7.2).

Growth properties HCS cells at passage 80 were collected by trypsinization and suspended in 10% FBS-DMEM/F12 medium (pH 7.2) to a density of 2.0×10^5 /mL with their growth properties measured as previously described [20,21]. In brief, HCS cells in every 3 culture flasks were counted every 12 hours and averaged (mean±SD, 3 parallel experiments). The growth curve was determined according to the average cell density.

Then, yield the growth curve on which the population doubling time of HCS cells based.

Chromosome analysis The chromosome specimens of the passage 80 HCS cells at logarithmic phase were prepared as described previously [20,21]. In brief, the HCS cells that at logarithmic phase were treated with 20μg/mL of colchicine at 37℃ for 10 hours. The cells were dislodged, centrifuged and treated with hypotonic 0.3% KCl for 30 minutes at 37℃, followed by fixation in fresh Carnoy's solution (methanol : glacial acetic acid = 3:1, v/v) for 10 minutes at room temperature. Finally, the cell suspension was dropped onto chilled glass slides and stained with Giemsa for 30 minutes. Chromosomes were counted for 300 metaphase cells and statistically analyzed.

Immunocytochemistry Immunocytochemistry assay were performed as described previously [20,21]. Briefly, HCS cells were plated in 24-well plates. At the logarithmic phase, the cells were washed with PBS solution, fixed with 4% paraformaldehyde for 10 minutes and treated with 0.3% Triton X-100 at room temperature for 10 minutes. The wells were blocked with 5% bovine calf serum (BCS, Invitrogen) containing D-Hanks solution at 37℃ for 30 minutes and incubated with mouse anti-human vimentin (1:50; Santa Cruz Biotechnology, Heidelberg, Germany), α-smooth muscle actin (1:100; Santa Cruz), Aldehyde dehydrogenase (ALDH) 3A1 (1:50; Santa Cruz), connexin 43 (1:200; Santa Cruz), integrin β1 (1:100; Santa Cruz),Na^+/K^+-ATPase (1:50; Santa Cruz), Ca^{2+}-ATPase (1:50; Santa Cruz) monoclonal antibody at 4℃ overnight according to manufacturer's

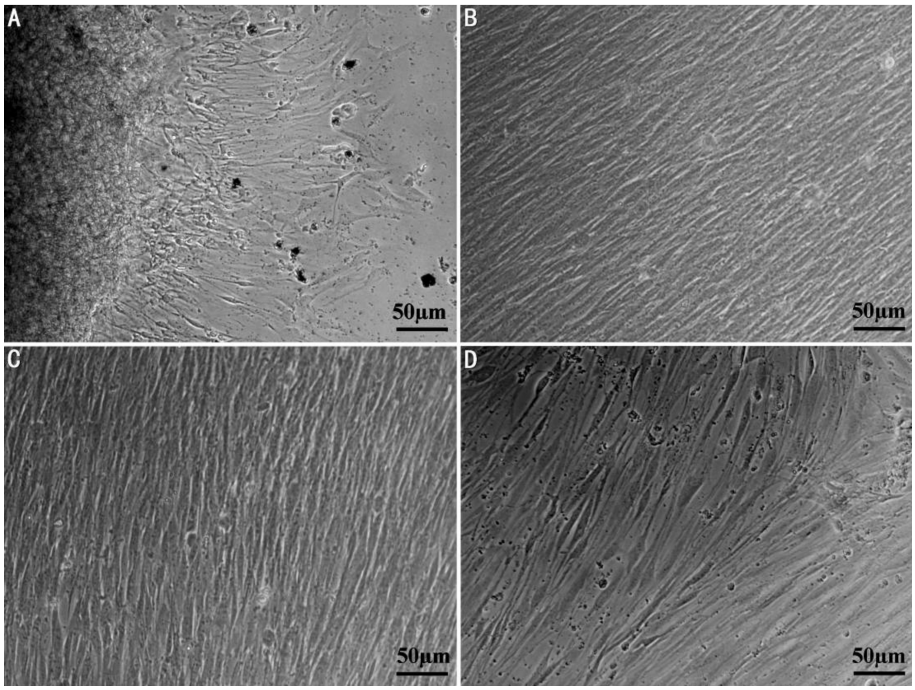

Figure 1 ***In vitro*** **culture of HCS cells** A: Primary cultures at day 3; B: A confluent monolayer formed 2 weeks later; C: At passage 60; D: At passage 80.

instructions. After incubated with fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Santa Cruz) at 37℃ for 1 hour, the wells were analyzed with a Ti-S inverted fluorescent microscope(Nikon, Tokyo, Japan). Omission of primary antibodies was used as controls.

Tumorigenesis Assay Male SPF BalB/c nude mice were maintained under specific pathogen-free conditions. Tumorigenesis assay of the HCS cells was performed as previously described [21]. HCS cells were harvested by a brief trypsinization, 1.0×10^7 viable cells were implanted into one of the forehand oxters each mice. In this group ten mice were used. As control, the other 10 were implanted with 1.0×10^7 HeLa cells. The tumorigenic status of the inoculated mice was monitored daily. Sixty days later, the mice were killed and the skin of the oxter of inoculated mice was surgically opened with tumorigenic status examined.

Preparation of aPCS Before the decellularization process, fresh porcine eyeballs were washed three times in sterile D-Hanks, and then remove the cornea by a pair of curved scissors along the limbus . The corneas were frozen at -80℃ for 3 hours and thaw at room temperature with three times. To remove the hereditary materials, the corneas were immersed in DNA-RNAase solution (0.5mg/mL) on an orbital shaker for 2 hours. Subsequently, the aPCS was washed three times with ddH_2O. Finally, the scaffolds were freeze-dried for 24 hours and stored at -80℃ before use.

Biocompatibility with aPCS HCS cells at logarithmic phase were collected with trypsinization at passage 80. And then were seeding on aPCS with the density of cell suspension adjusted to 5.0×10^5/mL with 10% FBS-DMEM/F12 medium (pH 7.2). Into each of an aPCS in a 24-well plate, 1.0mL cell suspension was plated and cultured at 37℃ with 5% CO_2. The medium was refreshed every day. Nine days later, the reconstructed HCS was examined with a JSM2840 scanning electron microscope (SEM; JEOL, Tokyo, Japan). The expression of vimentin was examined immunochemically as described above.

Statistical Analysis Data are expressed as mean ±SD (triplicates or decuplicates) and tested for statistical significance with ANOVA single factor.

RESULTS

In Vitro **Culture of HCS Cells** During primary culture, HCS cells migrated from corneal stromal pieces on day 3 (Figure 1A). They, all in fibroblastic shape, were highly transparent and grew into a 100% confluent monolayer in 2 weeks (Figure 1B). The HCS cells, maintaining their fibroblastic shape, grew and proliferated at a stable rate during subsequent subculture (Figure 1C). After subsequent subculture, a spontaneously continuous untransfected HCS cell line, designated as utHCSC01, has been established, which has been subcultured to passage 140 by now(Figure 1D). The population doubling time of the cell line was calculated to be 41.44 hours at passage 80 (Figure 2A).

Chromosome Analysis At passage 80, the chromosome analysis of HCS cells revealed that chromosomal aneuploidy, the number of chromosomes ranged from 39 to 52. The proportion of HCS cells with 46 chromosomes was about 61.72% (Figure 2B), and this suggest that the modal chromosome number of utHCSC01 cell line was still 46 with a normal karyotype (Figure 2C). HCS cells expressed

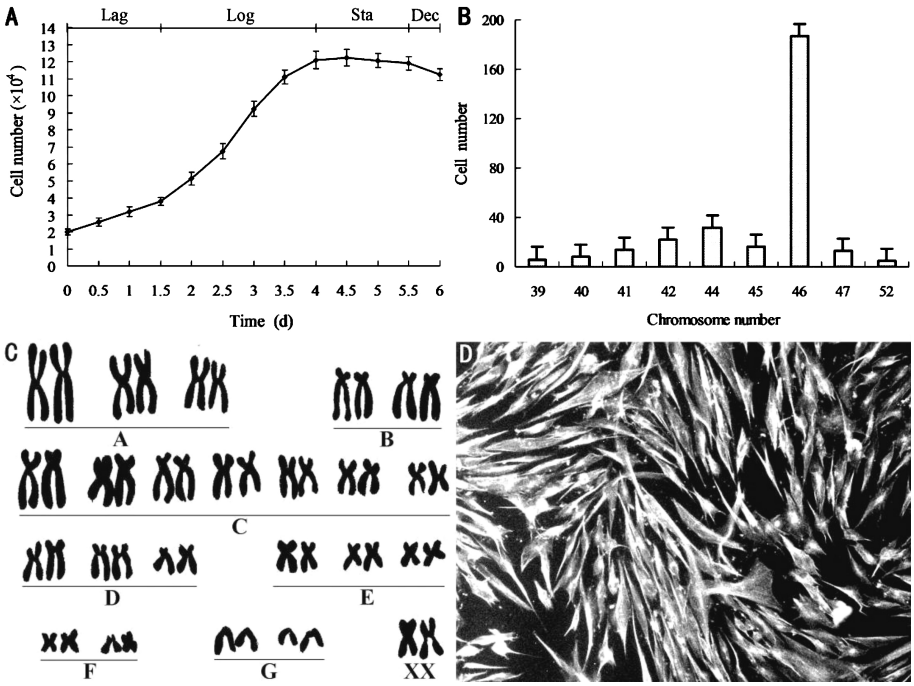

Figure 2 Properties of HCS cell line at passage 80 A: The growth curve (Lag, lag phase; Log, logarithmic phase; Sta, stationary phase; Dec, decline phase); B: Normal diploid karyotype; C: Chromosome aneuploidy; D: Vimentin expression.

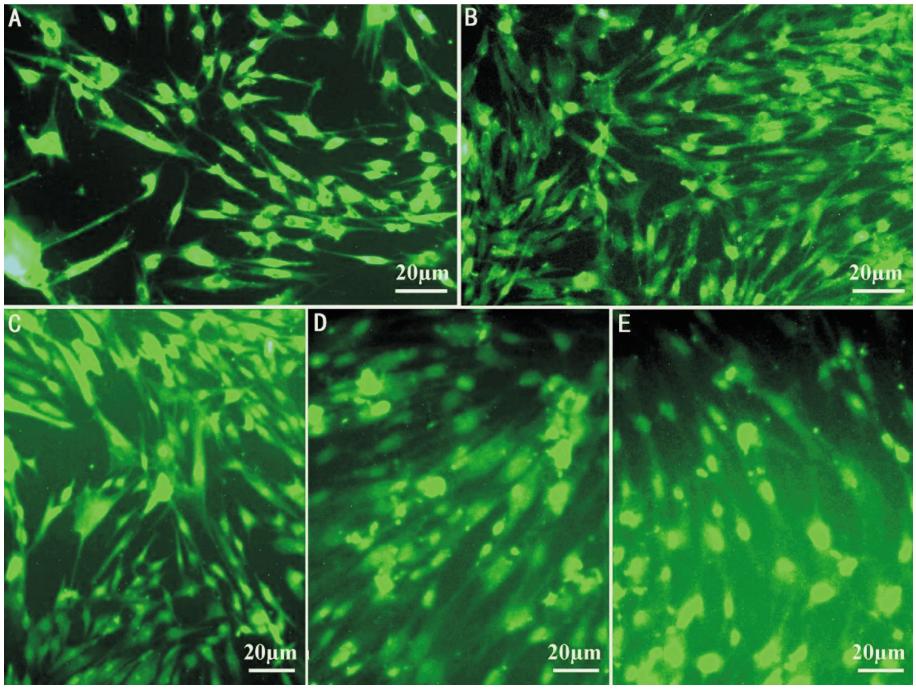

Figure 3 Immunofluorescence staining of HCS cells at passage 80 A: Connexin 43; B: Integrin β1; C: Aldehyde dehydrogenase (ALDH) 3A1; D: Na^+/K^+-ATPase; E: Ca^{2+}- ATPase.

vimentin (a specific marker of HCS cells) gene (Figure 2D), proving their HCS origin.

Immunocytochemistry At passage 80,HCS cells maintained expression of cell junction proteins including connexin 43 (Figure 3A) and integrin β1 (Figure 3B), and membrane transport proteins including aldehyde dehydrogenase (ALDH) (Figure 3C), Na^+/K^+-ATPase (Figure 3D) and Ca^{2+}-ATPase (Figure 3E), indicating that they reserved the potencies of forming cell junctions and performing membrane transport functions.

Tumorigenicity After passage 80 HCS cells being inoculated subcutaneously into 10 SPF BalB/c nude mice, no solid tumor was found on day 60 (Table 1). Whereas, progressively growing solid tumor was found in all 10 SPF BalB/c nude mice in 7 days after being inoculated with HeLa cells, indicating that the HCS cells were not tumorigenic.

Biocompatibility of HCS Cells with aPCS The passage 80 HCS cells migrated into aPCS scaffold 3 days after inoculation (Figure 4A). And the migrated HCS cells, in

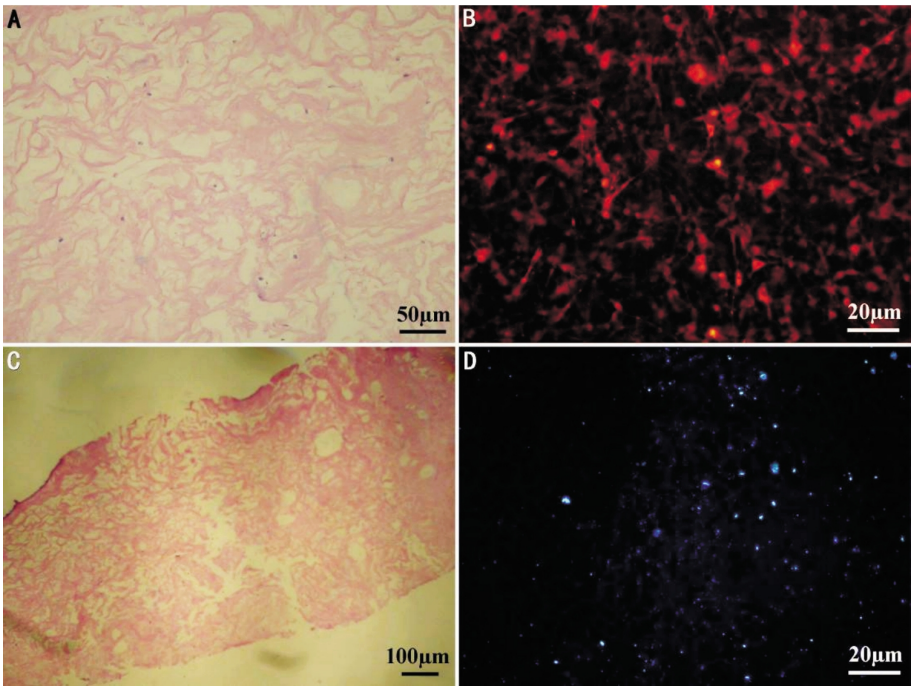

Figure 4 Characterization of HCS cells growing in aPCS A: HE staining; B: DiI fluorescence; C: HE staining; D: FITC fluorescent staining with vimentin antibody.

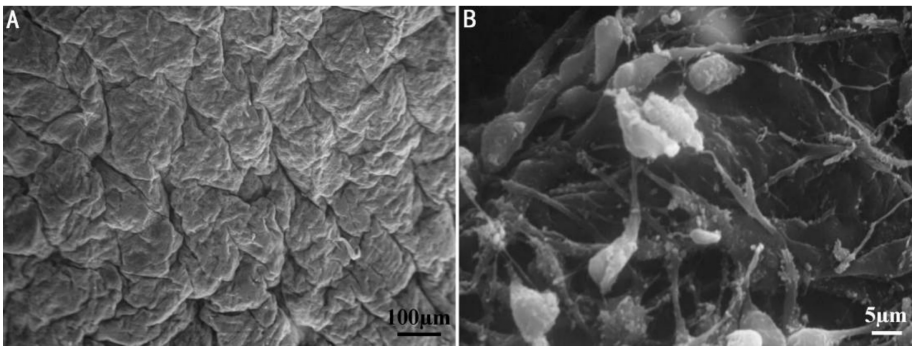

Figure 5 Scanning electron microscopic images of acellular porcine corneal stroma A: The surface of prepared aPCS; B: Fracture surface of HCS cell innoculated aPCS at day 9.

Table 1 Tumorigenesis assay of passage 80 HCS cells in SPF BALB/c nude mice

Inoculated cells	Total dose (cell/mouse)	Number of mice	Mortality (%)	Number of mice with tumor
HCS cells	1.0×10^7	10	0	0
HeLa cells	1.0×10^7	10	0	10

Note: tumors were identified surgically.

fibroblastic morphology (Figure 4B) and maintained positive vimentin expression (Figure 4D), grew very well inside the aPCS and a well-structured corneal stroma was reconstructed at day 9 (Figure 4C). Under SEM, the HCS cells were all in fibroblastic morphology and a lot of fibers were secreted from them (Figure 5).

DISCUSSION

HCS cell lines are necessary in studies of HCS cell proliferation, differentiation, apoptosis, cornea wound healing and TE-HCS reconstruction [22]. Even though numerous attempts have been made to obtain HCS cells in long-term culture, only 2 immortalized HCS cell lines have been established by gene transfection of SV40 T antigen and the human telomerase reverse transcriptase (hTERT) [19,23]. The effectiveness of these transfected HCS cell lines as potential research models has been hampered by genetic instability, abnormal phenotypes and tumorigenicity, precluding their effective use in studies of normal HCS cell biology and clinical HCS cell replacement [22]. No HCS cell line was established form human stromal tissues by now. Based on our previous experience in corneal cell line establishments [20,21], *in vitro* culture of HCS cells was performed by tearing off Bowman's membrane and Descemet's membrane in this study. Matrix attachment and proliferation of HCS cells has been successfully induced by replenishing culture medium with various supplements

including bFGF, EGF, chondroitin sulfate, carboxymethyl-chitosan and collagen IV. The successful induction of proliferation of HCS cells was consistent with the results obtained in cultured human corneal endothelial (HCE) cells [20] and human corneal epithelial (HCEP) cells [21].

With pure HCS cells and supplement-induced attachment and proliferation, a continuous untransfected HCS cell line, designated as utHCSC01, with the modal chromosome number of 46, had been successfully established and subcultured to passage 140 in this study. The cells proliferated actively and constantly with a population doubling time of 41.44 hours at passage 80, which was longer than those of HCE cell line (26.20 hours) [20] and shorter than those of HCEP cell line (45.42 hours) [21]. Besides, Positive expression of vimentin, a frequently used marker protein of HCS cells, combined with the fibroblastic morphology suggests that the utHCSC01 cell line established in this study was of a corneal stromal origin, consistent with the results obtained in previous studies of transfected HCS cells [19,23]. Taken together, these results indicated that established cell line was a pure HCS cell line without contamination of HCE and HCEP cells.

Cell junctions are crucial for HCS cells to communicate with each other and attach to extracellular matrix (ECM) for maintaining HCS structure and function, and HCS cells have been found to have connexin-43 and integrin β1 [24,25]. Connexin-43, as the main class of intercellular gap junction protein, plays an important role in corneal stromal intercellular communication [26]. Intergrins, a cell-ECM anchoring junction protein as the main class of cell adhesion receptors for the various components of the ECM, play an important role in interactions of corneal stromal cells to ECM [27]. In the present study, HCS cells at passage 80 still maintained stable expression of connexin-43 (an intercellular gap junction protein) and integrinβ1 (a cell-ECM anchoring junction protein), suggesting that the utHCSC01 cells still maintained normal capabilities of cell-cell communication and cell-ECM association by establishing intercellular gap junctions and cell-ECM junctions, and this property may be critical in the wound-healing process of cornea, especially in the avascular corneal environment [27]. This expression pattern of cell junction proteins in HCS cells is consistent with the results obtained from rabbit corneal stromal cells [24,25].

Function proteins play vital roles in maintaining HCS cell functions and in turn the structure and functions of HCS [22]. Membrane transport proteins of HCS cells, such as Na^+/K^+-ATPase and Ca^{2+}-ATPase, play crucial roles in membrane transport [20,28]. As demonstrated, Na^+/K^+-ATPase for Na^+ (out of cell) and K^+ (into cell) transport [20,29], and Ca^{2+}-ATPase for Ca^{2+} transport [30]. In this study, the HCS cells maintained positive gene expressions of Na^+/K^+-ATPase and Ca^{2+}-ATPase, indicating that the utHCSC01 cells still had potencies to carry out active transmembrane transport, consistent with the results obtained from human corneal endothelial cell line [20] and mouse corneal epithelium [30]. In addition, transparent cornea is directly exposed to ultraviolet (UV) radiation which might induce cellular damage and cell death by apoptotic and necrotic mechanisms, and ALDH, expressed specifically in corneal keratocytes but not fibroblasts or myofibroblasts, plays important roles in protecting HCS cells from damage of UV-induced oxidative stress [28]. In the present study, HCS cells reserved positive expression of ALDH, implying that the utHCSC01 cells maintained capabilities of oxidative stress protection, consistent with the results obtained from human keratocytes [31,32].

Oncogene transfected HCS cell lines, having latent potency of tumorigenicity and abnormal phenotypes, cannot be utilized in studies of HCS cells and *in vitro* reconstruction of TE-HCS [22]. Tumorigenic potency assay with SPF BalB/c nude mice in this study showed that the HCS cell line had no latent potency for tumorigenicity, suggesting that the utHCSC01 cell line could be safely used in studies of HCS cells and *in vitro* reconstruction of TE-HCS.

Excellent biocompatibility between HCS cells and scaffold carriers is a vital precondition for *in vitro* reconstruction of TE-HCS, an ideal equivalent of donated HCS [22,33]. Among the scaffold carriers used, aPCS has been proving to have good biocompatibility to HCS cells and successfully utilized in TE-HCS reconstruction [34,35]. In the present study, the fibroblastic cells from established HCS cell line migrated into aPCS scaffold 3 days after inoculation. The cells maintained positive vimentin expression and a well-structured corneal stroma with a lot of cell secreted fibers was reconstructed at day 9 (Figure 4C). These results indicated that the HCS cells could form integral HCS-like structures in aPCS and still maintained the inherent properties of corneal stromal cells, consistent with the results obtained from rabbit stromal keratocytes in aPCS [35]. The excellent biocompatibility to aPCS implied that the established HCS cell line might be feasible for reconstruction studies of TE-HCS.

In conclusion, a continuous untransfected and non-tumorigenic HCS cell line, with normal morphology, protein expression and excellent biocompatibility to aPCS, has been successfully established in this study. And the cell line provides a powerful tool for studies of proliferation and differentiation of HCS cells. It could also be used for *in vitro* reconstruction of TE-HCS, providing a promising method for the treatment of diseases caused by corneal stromal disorders. Studies on TE-HCS reconstruction and its rabbit transplantation are currently undertaken in our laboratory.

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