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Reconstruction of damaged corneal epithelium using Venus-labeled limbal epithelial stem cells and tracking of surviving donor cells

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ABSTRACT

Limbal epithelial stem cells are responsible for the self-renewal and replenishment of the corneal epithelium. Although it is possible to repair the ocular surface using limbal stem cell transplantation, the mechanisms behind this therapy are unclear. To investigate the distribution of surviving donor cells in a reconstructed corneal epithelium, we screened a Venus-labeled limbal stem cell strain in goats. Cells were cultivated on denuded human amniotic membrane for 21 days to produce Venus-labeled corneal epithelial sheets. The Venus-labeled corneal epithelial sheets were transplanted to goat models of limbal stem cell deficiency. At 3 months post-surgery, the damaged corneal epithelia were obviously improved in the transplanted group compared with the non-transplanted control, with the donor cells still residing in the reconstructed ocular surface epithelium. Using Venus as a marker, our results indicated that the location and survival of donor cells varied, depending on the corneal epithelial region. Additionally, immunofluorescent staining of the reconstructed corneal epithelium demonstrated that many $P63^{+}$ cells were unevenly distributed among basal and suprabasal epithelial layers. Our study provides a new model, and reveals some of the mechanisms involved in corneal epithelial cell regeneration research.

1. Introduction

The integrity and transparency of the cornea play a key role in vision. Limbal epithelial stem cells (LESCs) are responsible for the self-renewal and replenishment of the corneal epithelium (Beebe and Masters, 1996; Chen and Tseng, 1991; Cotsarelis et al., 1989; Pellegrini et al., 1999). Similar to other adult somatic stem cells, LESCs divide symmetrically to self-renew, or asymmetrically to produce daughter transit amplifying cells (TAC). These eventually become post-mitotic terminally differentiated (TD) cells (Notara and Daniels, 2008; Schlotzer-Schrehardt and Kruse, 2005). LESCs can be stimulated to initiate a series of activities that include cell division, migration and differentiation (Beebe and Masters, 1996; Majo et al., 2008). Congenital or acquired diseases, trauma and burns can result in partial or full LESC deficiency (LSCD). In such cases, the cornea becomes opaque, neovascularized and inflamed, which affects vision as the conjunctival epithelium invades across the limbus (Daniels et al., 2006). Cultured LESC therapy was originally described in 1997 by Pellegrini (Pellegrini et al., 1997). Clinical outcomes from different research groups have showed that greater than 75% of patients have been successfully treated using cultured LESC therapy (LSCT) (Baylis et al., 2011; Rama et al., 2010; Shortt et al., 2007).

Although cultured LESC transplantation has been shown to successfully repair the ocular surface, the mechanism(s) of therapy is unclear. Therefore, tracking of surviving donor cells is of significance in ophthalmology research. There are numerous technologies that can be applied to trace the survival of transplanted cells. Impression cytology (IC) has been previously used to examine morphological and pathological changes in epithelial cells of the ocular surface (Simon et al., 1992). Recently, IC was used for tracing surviving donor corneal epithelial cells (Wylegala et al., 2008). DNA polymorphisms and the polymerase chain reaction (PCR) have been used to examine surviving donor allografts for nearly 20 years (Daya et al., 2005; Henderson et al., 1997). The PCR method has the advantage of using few corneal cells, possessing high sensitivity, and results in minimal injuries to patients. Fluorescent *in situ* hybridization (FISH) can also be used to detect donor cells (Egarth et al., 2005). These aforementioned techniques are usually used in combination to ensure accuracy of the results (Henderson et al.,

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2001; Shimazaki et al., 1999). Tracking of donor cells can be conducted through sex-mismatching, DNA fingerprinting, detection of xenogeneic DNA and transgenic animal staining (Du et al., 2003; Majo et al., 2008).

We screened a Venus-labeled limbal epithelial stem cell (LESC-V) strain, and produced corneal epithelial sheets from them. We transplanted the Venus-labeled corneal epithelial sheets to goat models of limbal stem cell deficiency (LSCD). We investigated the distribution of donor corneal epithelial cells within the reconstructed ocular surface 3 months post-transplantation.

2. Materials and methods

2.1. Isolation and primary culture of goat LESCs

Fresh, whole corneal tissues were obtained from GuanZhong dairy goats (2–12 months old) based at Northwest A&F University (Shaanxi, China) animal farm. Goat corneal tissues were sectioned under sterile conditions. Corneal limbal tissues were used for LESC isolation, and some of the corneal tissues were prepared for hematoxylin and eosin (H&E) staining and for immunostaining. LESCs were isolated using a previously described method with some modifications (Qu et al., 2009). Limbal tissues were washed with phosphate-buffered saline (PBS) containing 100 IU/mL penicillin, 100 IU/mL streptomycin and 2.5 μg/mL amphotericin B (Hyclone, Thermo Scientific Corporation). After carefully removing excess corneal endothelium and conjunctiva, the remaining tissue was incubated with 1.2 IU of dispase II (Sigma–Aldrich, Shanghai, China) at 37 °C for 1.5 h. The limbal epithelial sheets were collected and separated into single cells with 0.125% trypsin (Sigma–Aldrich) and 0.02% EDTA (Gibco, Life Technologies Corporation, Beijing, China) at 37 °C for 5–7 min. The partially isolated LESCs were enriched with collagen IV (Sigma–Aldrich) using a previously described method (Li et al., 2005) with some modifications. Briefly, cells (5×10^4 cells/mL) were seeded onto collagen IV-coated (100 μg/mL) dishes and incubated at 37 °C for 20 min, and then the unattached cells were gently removed. The attached cells were cultured in fresh LESC medium [DMEM/F12 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific Corporation, Australia), 20 ng/mL epidermal growth factor (EGF; Peprotech, USA), 5 μg/mL bovine insulin (Sigma–Aldrich), 0.5 μg/mL hydrocortisone (Sigma–Aldrich), 180 nM adenine (Sigma–Aldrich), 2 nM 3, 5, 3′-triiodothyronine (Sigma–Aldrich), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 1.25 μg/mL amphotericin B (Hyclone, Thermo Scientific Corporation)] at 37 °C/5% CO_2, which was renewed every 2 days. When the primary cells reached 70–80% confluence, LESCs were treated with 0.125% trypsin/0.02% EDTA and partially enriched again according to the protocol above.

2.2. Establishment of LESC-V

Primary LESCs were transfected with pVenus, a vector encoding YFP which is a derivative of GFP. Lipofectamine™ 2000 (Invitrogen, Life Technologies) was used as the transfection reagent. Fluorescence in transfected cells was observed at 24 h post-transfection, following which cells were cultured with LESC culture medium supplemented with 500 μg/mL G418 (Geneticin, Life Technologies) for 1 month. Stably transfected positive clones displayed green fluorescence and were isolated with the assistance of an inverted fluorescence microscope (Leica Microsystems, Milton Keynes, UK). One of the stable transfectants was chosen for further proliferation, and designated LESC-V. Culture and passage of LESC-Vs was the same as for LESCs.

2.3. Total RNA extraction and RT-PCR assays

Total RNA was isolated from primary LESCs and LESC-Vs (1×10^6 cells) using a similarly described protocol (Li et al., 2005). The β-actin gene was used as an internal reference control, while the expression of other mRNAs was analyzed by reverse transcription polymerase chain reaction (RT-PCR) as previously described (Mi et al., 2008). First-strand cDNAs were synthesized from 0.5 μg of total RNA using a First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific Corporation, Beijing, China). Amplification by PCR was conducted with the use of various specific primers (Table 1).

2.4. Construction of Venus-labeled corneal epithelial sheets

Human amniotic membranes (HAMs) were obtained from healthy patients that underwent Cesarean section. The HAMs were prepared using an established method (Yang et al., 2008) with some modifications. HAMs were washed with PBS containing 100 IU/mL penicillin, 100 IU/mL streptomycin and 2.5 μg/mL amphotericin B until blood clots were no longer evident. The HAMs were then separated from the chorion by blunt dissection and cut into 10 cm × 10 cm pieces. HAMs were stored in DMEM (Gibco, Life Technologies) with glycerol [1:1 (v/v); Sigma–Aldrich] at −80 °C. Before production of the corneal epithelial sheets, cryopreserved HAMs were thawed at 37 °C and washed three times with PBS. The HAMs were incubated with 0.1% EDTA (Gibco, Life Technologies) at 37 °C for 2 h to remove epithelial cells. The denuded HAMs were cut into pieces with a 3-cm diameter and flattened, with the stromal side facing a nitrocellulose paper framework. The LESC-V (1×10^6 cells/mL) cells were seeded on the denuded HAM carrier in a 35-mm culture dish (Corning, Shanghai, China). The culture was submerged in LESC medium for 1 week and then exposed to air by lowering the medium level over a 2-week period. Cultures were incubated at 37 °C/5% CO_2 (Thermo Forma) for 21 days, and the medium was replenished every day.

2.5. Electron microscopy

Venus-labeled corneal epithelial sheets were examined by transmission electron microscopy (TEM, JEM-2000Ex, JEOL, Tokyo, Japan). Specimens were fixed in 2.5% glutaraldehyde, washed three times for 15 min in PBS, then fixed for 2 h in 2% aqueous osmium tetroxide. After washing three more times in PBS, specimens were passed through a graded ethanol series (50%, 70%, 90%, 95%, and 100%). For TEM, specimens were embedded in epoxy resin (Agar

Table 1
Oligonucleotide primer sequences used in the RT-PCR and qPCR assays.

Gene	Sequence (5′–3′)	Annealing temperature (°C)	Length (bp)
P63	Forward: CAGACTCAATTTAGTGAG Reverse: AGCTCATGGTTGGGGCAC	55	440
K3	Forward: GGCAGAGATCGAGGGTGTC Reverse: GTCATCCTTCGCCTGCTGTAG	59	145
K12	Forward: ACATGAAGAAGAACCACGAGGATG Reverse: TCTGCTCAGCGATGGTTTCA	60	150
$ABCG_2$	Forward: TGGAGTCATGAAACCTGGCCTC Reverse: AAAAGGACAGCATTCGCTGTGC	62	474
PCNA	Forward: AGTGGAGAACTTGGAAATGGAA Reverse: GAGACAGTGGAGTGGCTTTTGT	57	154
Venus	Forward: AAGTTCATCTGCACCACCG Reverse: AGCTCAGGTAGTGGTTGTCG	55	475
β-actin	Forward: CACGGTGCCCATCTACGA Reverse: CTTGATGTCACGGACGATTT	57	157