Limbal niche cells: a novel feeder cell for autologous cultivated oral mucosal epithelial transplantation

Chao-Ye Duan¹, Hua-Tao Xie¹, Xin-Yue Zhao¹ & Ming-Chang Zhang^{*,1}

¹Department of Ophthalmology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.

*Author for correspondence: mingchangzhang@hotmail.com

Aim: To establish a culture system using conspecific limbal niche cells (LNCs) as feeders for autologous cultivated oral mucosal epithelial transplantation (COMET). **Materials & methods:** Rabbit oral epithelial sheets, harvested from culture systems containing LNCs or 3T3 cells, were transplanted onto limbal stem cell-deficient rabbit eyes (COMET-3T3 or COMET-LNCs). **Results:** After COMET, corneas were relatively restored, with the exception of mild neovascularization in one cornea of the COMET-3T3 group. CD34 was detected in COMET-3T3 group corneas. Corneas of the COMET-LNCs group expressed high levels of PEDF and sFlt-1, but low levels of bFGF, compared with expression in COMET-3T3 corneas. **Conclusion:** The culture system containing conspecific LNC feeders could substitute for the 3T3 cell system and decrease the risk of neovascularization after COMET.

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The normal ocular surface is covered by corneal epithelium and conjunctival epithelium. The transitional zone between the cornea and conjunctiva is called the corneal limbus, which is considered the location where limbal stem cells (LSCs) reside [1]. The corneal limbus is also considered a barrier that prevents invasion of conjunctival epithelial cells and blood vessels to maintain the clarity of the cornea [2]. Different severe situations such as Stevens–Johnson syndrome, ocular cicatricial pemphigoid, chemical/thermal burns and other impairments can damage LSCs, destroying the barrier between the cornea and conjunctiva, and leading to LSC deficiency (LSCD). Consequently, the peripheral conjunctival epithelium invades toward the cornea, resulting in chronic corneal inflammation, scarring, conjunctivalization and vascularization with severe vision loss and other subjective symptoms [3–5].

Different types of surgical modalities have been developed to treat LSCD. Limbal autograft transplantation can be used to restore the corneal surface in this disease [6]. It has been reported as a safe procedure because LSCs can repopulate the donor sites after limbus removal for transplantation [7]. However, it cannot be applied in cases of bilateral LSCD, such as Stevens–Johnson syndrome and ocular cicatricial pemphigoid [8]. Limbal allograft transplantation can be performed to rescue the ocular surface of unilateral or bilateral LSCD [9,10], but it requires extended immunosuppressive therapy, which can lead to eye and systemic complications [9]. In addition, the number of donor organs might not meet demand in some areas [11]. With improved stem cell technology, cultivated limbal epithelial transplantation was described by Pellegrini *et al.* in 1997 [12] to deal with unilateral situations. In cases of bilateral LSCD, oral mucosal epithelial cells (OMECs) that can be obtained easily and safely were reported to be an alternative cell source for ocular surface reconstruction [13]. Moreover, autologous cultivated oral mucosal epithelial transplantation (COMET) is not restricted by donor tissue and immunosuppression. Nevertheless, various degrees of peripheral corneal neovascularization were detected in many cases, even after successful COMET [14–18].

As reported, the colony forming efficiency of limbal epithelial cells co-cultured with feeder cells was higher than that of limbal epithelial cells without feeders [19]. Therefore, the use of feeder cells seems to be vital for engineering epithelial sheets that contain progenitor cells [20]. Studies have demonstrated that in the presence of 3T3 feeder cells,

cultivated oral mucosal epithelial cells (COMECS) express fewer anti-angiogenic factors, but more pro-angiogenic factors than cultivated corneal epithelial cells, which might play a role in the observed neovascularization after COMET [21–23]. The stem cell niche is believed to play a critical role in the regulation of stem cells [24]. The limbal niche has been proposed to regulate the fate of LSCs [25], and limbal niche cells (LNCs) are considered one of several components of the limbal niche [26]. Moreover, we have successfully isolated and expanded LNCs *in vitro* [27,28].

In this present study, we cultured rabbit OMECs with conspecific LNCs or mouse-derived 3T3 cells on a denuded amniotic membrane (dAM). We then performed COMET onto the ocular surfaces using a rabbit model of induced LSCD and evaluated the characteristics of grafted corneas.

Materials & methods

Animals

Healthy Japanese white rabbits of either sex (weighing approximately 2–2.5 kg) were purchased from the Laboratory Animal Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). Animal-related activities adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (Permit Number: S641).

LSCD induction

LSCD was induced in the right eyes of nine rabbits as previously reported [29–31] with some modifications. Briefly, the procedures were performed as follows: rabbits were anesthetized with an intramuscular injection of 0.2 ml/kg compound xylazine (Nanjing Agricultural College, China) and received topical anesthesia with a drop of oxybuprocaine hydrochloride eye drops (Santen Pharmaceutical Co., Ltd, Japan). Then, corneal epithelial cells were removed. Next, lamellar excision, which extended to approximately 2 mm outside the limbal zone at an approximate depth of 30% of total limbal thickness, was performed, and the conjunctival tissue proximal to the limbus was excised. Antibiotic eye drops were administered after injury for 7 days, 3–4 times per day. Ophthalmological observations were conducted by slit-lamp microscopy and fluorescein staining. Photographs of rabbit corneas were taken after operation.

Preparation of dAM & cells

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human placentas were obtained immediately after cesarean deliveries. The amniotic membrane (AM) was separated from the chorion by blunt dissection, treated as described previously [32], and then stored at -80°C in Dulbecco's modified Eagle's medium (HyClone, UT, USA) with glycerol (Union Hospital, Wuhan, China) at a 1:1 (vol/vol) ratio. Before use, the AM was thawed and washed three times with phosphate-buffered saline containing 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin and 5 $\mu\text{g}/\text{ml}$ amphotericin B. The AM was then submerged in Dispase II (Roche, IN, USA) for 5 min at 37°C and the epithelial cells were gently scraped off with a sterile brush.

After the rabbits were sacrificed with intravenous sodium pentobarbital (200 mg/kg), LNCs were isolated and expanded essentially as described previously by our group [27,28]. Healthy eyeballs were removed, and the corneoscleral rims were cut into small segments, which was followed by digestion with 1 mg/ml collagenase A (Roche) at 37°C for 18 h, and with 0.25% trypsin–EDTA solution (Gibco, NY, USA) at 37°C for 20 min. Then, the cell suspensions were seeded onto six-well plates coated with 5% diluted Matrigel (60 $\mu\text{l}/\text{cm}^2$; Corning, MA, USA) in modified embryonic stem cell medium [17]. All experiments were performed with purified LNCs of passage (P)3 and P4.

Oral mucosal biopsy specimens were removed from sedated rabbits as described [30] and COMECs were obtained using a similar method as previously reported [13,33]. Briefly, oral biopsies were digested with 10 mg/ml Dispase II at 37°C for 1 h. Subsequently, the epithelial cell sheets were peeled off and treated with 0.25% trypsin–EDTA solution at 37°C for 20 min to generate single cells. Then, cells were seeded onto culture inserts (3×10^5 cells/insert), on which the original membrane was manually replaced with dAM (Figure 1A–D), in Keratinocyte growth medium 2 (KGM 2; PromoCell, Heidelberg, Germany), and co-cultured with confluent conspecific LNCs or 3T3 cells (American type culture collection) that were treated with mitomycin C (Sigma–Aldrich, MO, USA). Culture medium was changed on day 3 after seeding for the first time and every 2 days thereafter. The feeder cells were renewed every 3 days. After epithelial cells reached confluence, the culture medium was lowered to let the cells

Figure 1. Preparation of modified culture model. (A) The original membrane of culture insert was peeled off by forceps. (B) The dAM was prepared. (C, D) The dAM was fixed to the bottom of the insert using a sterile rubber band. (E) Schematic illustration of culture system. dAM: Denuded amniotic membrane; LNC: Limbal niche cell.

Table 1. Primer sequences used for PCR.

Name	Primer	Sequence	Size (bp)
<i>β-actin</i>	Sense	AGCAAGCAGGAGTATGACGAGT	134
	Antisense	ATGCCAATCTCGTCTCGTTTCT	
<i>Vim</i>	Sense	GCAGTACGAAAGTGTGGCTG	197
	Antisense	GGGACTCGTTAGTTCCTTAAGC	
<i>CK12</i>	Sense	GGTTCATTGAAAAGAGCGGGG	189
	Antisense	CGCAGTAGTCGCCTTCAGTT	
<i>bFGF</i>	Sense	GGGTTTCTTCCTGCGTATCCA	284
	Antisense	GCCCAAGTTCGTTTCAGTGCC	
<i>PEDF</i>	Sense	CCAACTTCGGCTATGACCTGT	147
	Antisense	GAGCCCGGTGAATGATGGA	
<i>sFlt-1</i>	Sense	GCAAACAAGTAAGAGGGCTTCG	217
	Antisense	CTGCTGTCTCCGATGTTTACA	

cultured at the air–liquid interface (airlifting) for another 2 weeks, which was followed by transport of the AM carriers to a laboratory animal operating room for transplantation.

RT-PCR

Total RNA from LNCs of P0 and P3 was extracted with TRIzol reagent (Thermo Fisher Scientific, CA, USA), and cDNA was synthesized using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Then genes encoding *Vim* and *CK12* (see Table 1 for primers used) were detected by RT-PCR with gene amplifiers (EDC-810, EASTWIN, China). The RT-PCR profile consisted of 10 min of initial activation at 95°C followed by 40 cycles of 30s denaturation at 95°C, and 30s annealing and extension at 60°C.

Immunocytochemistry

Single LNCs of P0 and P3 were transferred onto glass slides for immunocytochemistry using cytospin (Shandon CytoSpin 3 Cyto centrifuge, Thermo Scientific, MA, USA) at $1200 \times g$ for 10 min. Then the 4% formaldehyde-fixed cells were treated with 0.5% Triton X-100 for 20 min at room temperature and blocked with 5% bovine serum

Table 2. Antibodies used for immunofluorescent staining.

Antibodies	Sources	Dilutions
Vim	Invitrogen (MA5-11883)	2 µg/ml
CK12	ABCAM (Ab185627)	1:100
Goat antimouse IgG (H+L) secondary antibody, Cy3 conjugate	BOSTER (BA1031)	1:100
Goat antirabbit IgG (H+L) secondary antibody, FITC conjugate	BOSTER (BA1105)	1:100
MUC5AC	LifeSpan BioSciences (LS-C390690)	1:50
CD34	BIOSS (BS-2038R)	1:100
α-SMA	ABCAM (Ab7817)	1:500
Cy3-AffiniPure goat antimouse IgG (H+L)	Jackson ImmunoResearch (115-165-003)	1:300
Cy3-AffiniPure goat antirabbit IgG (H+L)	Jackson ImmunoResearch (111-165-003)	1:300
FITC: Fluorescein isothiocyanate.		

albumin (BSA, Roche). After that, the cells were sequentially incubated with specific primary antibodies (overnight at 4°C) and corresponding secondary antibodies (1 h at 37°C; see Table 2 for information on antibodies). Nuclear counterstaining was achieved by 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Tokyo, Japan) staining. Images were taken using a fluorescence microscope (NIKON, Tokyo, Japan).

COMET

4 weeks after LSCD induction, corneal opacity, conjunctivalization and neovascularization were observed and the tissue-engineered cell sheets cultured with different feeder cells were transplanted onto the damaged ocular surfaces as described previously [30] with minor modifications. Conjunctival and scar tissue were removed from the cornea to re-expose the corneal stroma. Subsequently, the transplantable cell sheet was directly placed over the transparent stromal bed, along with the dAM carrier and sutured to the corneal surface with 10-0 nylon sutures (Alcon Laboratories, Inc., TX, USA). Then, tarsorrhaphy was performed with 5-0 nonabsorbable sutures (Ethicon, Inc., OH, USA) and the sutures were removed 2 weeks later. The rabbits were chosen randomly for sham surgery, which consisted of the same steps as COMET, except COMECs were not placed on the dAM. Eyes receiving different surgical treatments were termed the COMET-3T3, COMET-LNCs and SHAM groups, and each group contained three rabbits randomly assigned.

All eyes after surgery were treated with tobramycin and dexamethasone eye drops (Alcon Laboratories, Inc.) three-times per day for approximately 1 week and were observed with slit-lamp microscope. After 8 weeks, the rabbits were sacrificed as described previously herein, and the eyes were enucleated for further studies.

Immunohistochemistry

Corneas of healthy rabbits, the SHAM group, the COMET-3T3 group and the COMET-LNCs group were examined by immunofluorescence. Briefly, paraffin sections (4 µm thick) were deparaffinized and rehydrated and the antigens were unmasked in citric acid buffer (PH 6.0) via the microwave antigen retrieval technique. After blocking with 5% BSA, specific primary antibodies (overnight at 4°C) and corresponding secondary antibodies (50 min at room temperature) were sequentially incubated with the sections. Incubation with nonimmune immunoglobulin G, in place of primary antibodies, was used as a negative control (Supplementary Figure 1). The sections were counterstained with DAPI and photographed. Detailed information for the antibodies is listed in Table 2. The goblet cells were investigated by Periodic acid schiff (PAS) staining.

RT-qPCR

Total RNA of corneas treated with two different COMET approaches was extracted and reverse transcribed to cDNA as described previously herein. The expression of *bFGF*, *PEDF* and *sFlt-1* mRNA was investigated in accordance with the manufacturer's protocol using the StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA) as follows: 1 cycle at 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers used are listed in Table 1. The expression level of the housekeeping gene *β-actin* was used as an internal control; relative gene expression was analyzed by the $2^{-\Delta\Delta C_t}$ method [34].

Table 3. Antibodies used in western blotting.

Name	Sources	Dilutions
GADPH	Servicebio (GB12002)	1:1000
bFGF	Bioss (BS-0217R)	1:1000
PEDF	Santa Cruz (SC-390172)	1:1000
sFlt-1	Santa Cruz (SC-271789)	1:1000
Goat antirabbit IgG-HRP	Servicebio (GB23303)	1:3000
Goat antimouse IgG-HRP	Servicebio (GB23301)	1:3000

Western blotting

The cornea samples of different groups were lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China) supplemented with phenylmethanesulfonyl fluoride (PMSF; Beyotime Biotechnology, Shanghai, China). Then, the lysates were denatured and the same amount of protein in each sample was subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene fluoride membranes (PVDF; Millipore, Darmstadt, Germany), and treated with 5% skim milk for 1 h at room temperature. Then, the membranes were incubated with specific primary antibodies and respective secondary antibodies (listed in Table 3). GAPDH was used as an endogenous control. The immunoreactive proteins were visualized with an enhanced chemiluminescence kit (ECL; PerkinElmer, MA, USA), which was followed by digitizing with AlphaEase FC software (Alpha Innotech, CA, USA).

Statistics

Data were obtained from three separate experiments and analyzed by performing two-tailed unpaired t-tests (GraphPad Prism 5.0 software, CA, USA). Results of comparisons are expressed as the means $\pm$ standard error of the mean; $p < 0.05$ was considered statistically significant.

Results

Isolation & purification of LNCs

LNCs were isolated and expanded successfully. After subculturing, most LNCs were spindle-shaped at P3 (Figure 2A). Results of RT-PCR (Figure 2B) and immunocytochemistry (Figure 2C) showed that LNCs of P0 expressed Vim (marker of mesenchymal cells) and CK12 (marker of corneal epithelial cells); however, LNCs of P3 expressed Vim only, indicating that the epithelial cells were excluded and that the LNCs were purified.

Culture of COMECs using different co-culture systems

OMECs were cultivated using the culture model described previously herein (Figure 1E). After nonadherent cells were removed by changing the culture medium, cobblestone-like cells were observed in both systems (Figure 3A & B). The cells were tightly adherent to the dAM and reached confluence within 10 days (Figure 3C & D). After two additional weeks of airlifting culture, tissue-engineered cell sheets were obtained from both culture systems, and these were found to have two or three layers (Figure 3E & F), which are similar to normal corneal morphology (Figure 3G).

Establishment of LSCD model

After the induction of LSCD, images of the cornea with or without fluorescein staining were taken at different time points (Figure 4). Within 1 week of surgical injury, fluorescein staining covered the entire corneal surface. Then, the stained area became smaller over time and the corneal opacity, conjunctivalization and neovascularization gradually appeared without returning to normal (Figure 4A). Hematoxylin and eosin staining of corneal cross-sections showed that compared with the normal cornea, the structure of the LSCD cornea was disordered, and inflammatory cells, goblet cells and neovascularization were observed in the stroma (Figure 4B).

Reconstruction of the corneal surface

The dAMs, with or without autologous epithelial sheets, were successfully transplanted onto diseased ocular surfaces, and images representing ocular recovery were obtained (Figure 5). Ocular surfaces in the SHAM group failed to repel fluorescein staining, whereas those of the COMET-3T3 and COMET-LNCs groups were not stained by fluorescein dye immediately after transplantation. 4 weeks after surgery, corneal transparency in the COMET-3T3

Figure 2. Successful isolation and purification of limbal niche cells. Most LNCs were spindle-shaped at passage (P3) (A) RT-PCR showed that LNCs of P0 expressed *Vim* and *CK12*, whereas those of P3 expressed *Vim* only (B) *Vim*⁺ (red fluorescence) and *CK12*⁺ (green fluorescence) cell were investigated in P0 LNCs, while only *Vim*⁺ cells were detected in LNCs of P3 by immunocytochemistry (C) Scale bars: A: 50 μ m; C: 20 μ m. LNC: Limbal niche cell.

and COMET-LNCs groups improved with few epithelial defects. However, SHAM corneas exhibited unclarity, regrowth of blood vessels and larger areas of epithelial defects. 8 weeks after transplantation, ocular surfaces in the COMET-3T3 and COMET-LNCs groups were well reconstructed with a clear and smooth appearance, with the exception of mild peripheral neovascularization observed at the 1 o'clock position in one cornea of the COMET-3T3 group. Marked neovascularization and opacity were observed in all SHAM corneas at that time.

COMET-LNCs group corneas exhibit decreased neovascularization

Immunohistochemistry analysis was used to further document the effects of successful COMET. We compared biomarker expression profiles among corneas of normal rabbit, SHAM, COMET-3T3 and COMET-LNCs groups (Figure 6). PAS-positive goblet cells were found in the SHAM corneas. In addition, MUC5AC, often used as a goblet cell marker, was markedly expressed in corneas of the SHAM group. CD34, a hematopoietic progenitor marker [35], stained corneas of the SHAM group and, to a lesser extent, corneas of the COMET-3T3 group, implying that neovascularization occurred in these groups. Immunostaining for α -SMA was performed to investigate pathological fibrotic tissue invasion in the cornea [36,37], and this was positive in SHAM corneas only.

Figure 3. Establishment of transplantable cultivated oral mucosal epithelial cell sheets. Oral mucosal epithelial cells co-cultured with 3T3 cells (A, C) or LNCs (B, D) at day 3 (A, B), reached confluence within 10 days (C, D). After two additional weeks of airlifting, the oral mucosal epithelial cell sheets cultured with 3T3 cells (E) or LNCs (F) were harvested and were shown to consist of two to three layers. These cell sheets were similar in morphologic appearance to the normal cornea (G). Scale bars: A–D: 30 μm ; E–G: 12.5 μm . LNC: Limbal niche cell.

Expression of angiogenesis-related factors

To preliminarily investigate the mechanism underlying phenotypic differences in corneas between the two COMET groups, we compared the expression of bFGF, PEDF and sFlt-1. Transcription of these factors was measured by RT-qPCR (Figure 7), and results revealed that corneas of the COMET-LNCs group expressed higher levels of *PEDF* ($p < 0.05$) and *sFlt-1* ($p < 0.05$), but lower amounts of *bFGF* ($p < 0.05$), than corneas of the COMET-3T3 group. Raw data were listed in Supplementary Table 1. Western blotting was used to confirm the expression profiles of these angiogenesis-related factors at the protein level (Figure 8), and these results were consistent with those of RT-qPCR assays; differences in protein expression were also significant (all $p < 0.05$). Raw data are available in Supplementary Table 2.

Discussion

For decades, OMECs have been reported to be an alternative cell source for the reconstruction of the injured ocular surface, based on clinical and experimental studies [13,17,38,39]. Most of these used mouse-derived 3T3 cells as feeder cells to generate transplantable cell sheets. However, even though there has been no report regarding the possible side effects of using xenogeneic feeder cells, there is the possibility of infection or pathogen transmission from other species [20]. A conspecific feeder cell might be a safer choice for harvesting stratified epithelial sheets. Peripheral corneal neovascularization was observed after the transplantation of COMECs that were co-cultured with 3T3 cells, which hindered the application of this technique. Thus, some changes need to be made to ameliorate these adverse effects. Since the stem cell niche plays a critical role in the regulation of stem cells, we hypothesized the homogenous feeder cells derived from the limbal niche could change the characteristics of OMECs. It has been reported that the dermal fibroblast, bone marrow-derived mesenchymal cells and conjunctival fibroblasts can be used as feeder cells for the cultivation of OMECs [40–42]; however, components of the limbal niche are not present in these culture systems. The limbal niche is a specialized microenvironment of LSCs that contains different cell types and adjacent

Figure 4. Establishment of limbal stem cell-deficiency model. After the ocular surfaces were damaged, eyes were visualized using a slit lamp biomicroscope without (top) and with (bottom) fluorescein dye staining (A). Hematoxylin and eosin staining showed that the structures of LSCD corneas were disordered; further, inflammatory cells infiltrated the stroma, the presence of goblet cells (black arrows) and neovascularization (blue arrows) was also confirmed (B). Scale bars: 100 μ m. LSCD: Limbal stem cell deficiency.

cellular and extracellular components [25]. It was previously shown that collagenase can successfully isolate LSCs and a subset of mesenchymal cells [43]. According to the anatomical location, LSCs might closely interact with cells in the underlying limbal stroma. Therefore, we speculated that these stromal mesenchymal cells may serve as niche cells and continued to use the name 'LNCs' that used in our previous papers [27,28,43]. However, the definition and extraction methods of LNCs have not been unified [26,27]. Further studies are required to compare the differences of 'LNCs' extracted by various methods and to establish a unified definition and a standard method of extraction for LNCs. As a major constituent of the limbal niche, LNCs have been shown to regulate the fate of LSCs and maintain the growth of corneal epithelial cells [25,44]. What's more, LNCs were proved to possess greater capacity to support the limbal epithelial stem/progenitor cells than limbal stromal cells (another important cellular population in LSCs microenvironment) [26]. Therefore, we used LNCs to co-culture OMECs, and investigated the phenotypic differences between this new culture system and the commonly used culture system employing 3T3 feeder cells.

During the cell culture phase, we modified the commercial culture insert to obtain a dAM-fixing device for cultivating OMECs (Figure 1). The dAM fixed on the bottom of insert did not float, break, bend or curl easily when changing the culture medium, which offered a stable scaffold for OMEC growth. These cells co-cultured with conspecific LNCs showed no difference compared with those co-cultured with 3T3 cells in terms of cell shape

Figure 5. Representative images of the grafted cornea without (top) and with (bottom) fluorescein dye staining. After surgery, the ocular surfaces of the SHAM group stained positive, whereas staining was negative on the ocular surfaces of the two COMET groups. 4 weeks later, the visibility of the iris and pupil increased in eyes of both COMET groups, with small areas of fluorescein staining. 8 weeks after grafting, SHAM corneas showed obvious opacity and neovascularization, whereas surfaces of the COMET groups were almost clear and smooth. One cornea of the COMET-3T3 group showed mild peripheral neovascularization at the 1 o'clock position (black arrows). Each group contained three rabbits. COMET: Autologous cultivated oral mucosal epithelial transplantation; LNC: Limbal niche cell.

or growth rate (Figure 3A–D). After using the airlifting technique, multilayered epithelial cell sheets were harvested (Figure 3E & F), and the number of cell layers in both culture systems was similar. These findings demonstrated that LNCs could be a novel and practical feeder cell to culture OMECs.

A model of LSCD induction was utilized, via kerectomy and limbectomy, according to a previous study [33]. 4 weeks after surgical induction, the damaged corneas were covered with blood vessels and opaque tissues and remission was not observed. Hematoxylin and eosin staining of LSCD corneal cross-section showed the structure of the irregular corneal stroma, inflammatory cells, goblet cells and blood vessels. These results implied that the LSCD model was successfully established.

The appearance of goblet cells, blood vessels and fibrotic tissue comprise a basic feature of LSCD [45], which can be confirmed by PAS staining and the expression of MUC5AC, CD34 and α -SMA. After transplantation, the ocular surfaces were assessed for 8 weeks to detect changes in the corneas of different groups, PAS staining and immunostaining for MUC5AC, CD34 and α -SMA was performed to assess corneal restoration. Results demonstrated that corneal surfaces in the COMET-3T3 and COMET-LNCs groups were gradually restored. Pathologic signs of LSCD recurred in corneas of the SHAM group, or were even worse than those before surgery. Of note, 8 weeks after COMET, mild neovascularization was observed on one cornea of the COMET-3T3 group by slit-lamp microscopy. In addition, CD34, a marker often used for the detection of vascular endothelial cells [46,47], was detected on corneas of the COMET-3T3 group. This is consistent with the clinical observation that corneal neovascularization occurs after traditional COMET [14–18]. Nevertheless, no signs of neovascularization were detected by slit-lamp microscopy or immunohistochemistry on corneas of the COMET-LNCs group.

Previous studies implied that an imbalance in the expression of angiogenic factors in COMECs co-cultured with 3T3 cells [21–23] could be the reason as to why blood vessels recur after COMET. Our RT-qPCR and western blotting results showed that corneas of the COMET-LNCs group expressed high levels of anti-angiogenic factors (PEDF [48]

Figure 6. Representative immunohistochemical staining for angiogenesis-related factors after autologous cultivated oral mucosal epithelial transplantation. PAS, MUC5AC, CD34, and α -SMA staining was performed on corneas of normal rabbit, SHAM, COMET-3T3 and COMET-LNCs groups. PAS staining of goblet cells was detected in the SHAM corneas only. MUC5AC was expressed in the corneas of the SHAM group, but was not expressed in normal corneas or those after COMET. CD34 was markedly expressed in SHAM corneas and weakly expressed in corneas of the COMET-3T3 group. α -SMA was expressed in SHAM corneas.

Scale bars: 100 μ m.

COMET: Autologous cultivated oral mucosal epithelial transplantation; LNC: Limbal niche cell; PAS: Periodic acid schiff.

Figure 7. Comparison of gene expression in corneal specimens of different groups. Corneas of the COMET-LNCs group expressed low levels of *bFGF* ($p = 0.0092$), but high levels of *PEDF* ($p = 0.0259$) and *sFlt-1* ($p = 0.0018$), compared with expression in COMET-3T3 corneas. β -actin was used as an internal control, and the data were expressed as means $\pm$ standard error of the mean.

* $p < 0.05$.

COMET: Autologous cultivated oral mucosal epithelial transplantation; LNC: Limbal niche cell.

Figure 8. Comparison of the expression of angiogenesis-related proteins between corneas of different groups. Immunoreactive bands of bFGF, PEDF and sFlt-1 were detected by western blotting and normalized to GAPDH levels (A). Comparison of the relative expression of bFGF (B) ($p < 0.0001$), PEDF (C) ($p = 0.0001$), and sFlt-1 (D) ($p = 0.0396$), were made between corneas of COMET-3T3 and COMET-LNCs groups. The results are expressed as means $\pm$ standard error of the mean.

* $p < 0.05$.

COMET: Autologous cultivated oral mucosal epithelial transplantation; LNC: Limbal niche cell.

and sFlt-1 [49]), but low levels of a pro-angiogenic factor (bFGF [50]), compared with expression in corneas of the COMET-3T3 group; this confirmed the previous observation and indicated that the culture system containing LNCs could reduce the risk of postoperative neovascularization. It was previously shown that different levels of biomarkers expressed by *in vitro*-cultivated cells were related to the different feeder layers with which these cells were cultured [41,42,44]. Similarly, in our study, the expression levels of angiogenesis-related factors differed when using distinct feeder cells. Recently, Eslani *et al.* showed that the secretome of corneal-derived mesenchymal stromal cells could inhibit human umbilical vein endothelial cell tube and sprout formation [51]. Based on these studies, we assumed that LNCs affected COMECs by secreting angiogenesis-related factors or other organic molecules. However, the specific mechanism remains to be identified.

In summary, we present a new culture system consisting of conspecific LNC feeders to obtain transplantable oral mucosal epithelial sheets *in vitro*. COMECs cultivated in this new system were as robust and stratified as those cultivated with 3T3 feeder cells. Moreover, we successfully restored LSCD corneas by COMET (when transplantable epithelial sheets were co-cultured with 3T3 cells or LNCs). Further, the use of LNCs as feeders could reduce the risk of neovascularization after COMET. We believe that allogeneic LNCs, derived from a safer cell source and possessing a distinct therapeutic advantage, represents a superior feeder cell for cultivating OMECs to reconstruct the ocular surface after LSCD. These experimental findings might provide the basis for an improved

ex vivo culture system and the more widespread application of COMET. However, further studies are still needed to investigate the specific mechanism.

Summary points

- Autologous cultivated oral mucosal epithelial transplantation (COMET), a feasible treatment for limbal stem cell deficiency (LSCD), avoids restrictions associated with donor tissue and immunosuppression.
- We aimed to establish a culture system using conspecific limbal niche cells (LNCs) as feeders to avoid xenogeneic infection and postoperative neovascularization.
- Here, we established LSCD models using Japanese white rabbits.
- Morphologically similar, autologous oral epithelial sheets, harvested from culture systems containing rabbit LNCs or 3T3 cells, were transplanted onto corneas (COMET-3T3, COMET-LNCs and SHAM groups).
- 8 weeks after COMET, corneas were restored and exhibited relatively clear and smooth appearances, with the exception of mild neovascularization in the cornea of one COMET-3T3 group animal.
- MUC5AC and α -SMA staining was observed only in corneas of the SHAM group, whereas marked CD34 staining was identified in sham corneas and to a lesser extent COMET-3T3 group corneas.
- Based on RT-qPCR and western blotting, corneas of the COMET-LNCs group expressed high levels of anti-angiogenic factors (PEDF and sFlt-1), but low levels of a pro-angiogenic factor, compared with expression in COMET-3T3 corneas ($p < 0.05$).
- Thus, the new culture system containing conspecific LNC feeders could substitute for the commonly used 3T3 cell feeder system, and could decrease the risk of neovascularization after COMET.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/rme-2018-0122

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Ethical conduct of research

Animal-related activities adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology. This article does not contain any studies with human participants performed by any of the authors.

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CY Duan and MC Zhang conceived and designed the experiments. XY Zhao contributed reagents, materials and analysis tools. CY Duan performed the experiments. HT Xie analyzed the data. CY Duan wrote the paper. All authors read and approved the final manuscript.

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