

Umbilical Cord Lining Stem Cells as a Novel and Promising Source for Ocular Surface Regeneration

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Abstract The stem cells involved in renewal of the corneal epithelium are located in the basal region of the limbus, a narrow transition zone surrounding the cornea. In many ocular surface disorders loss of these stem cells results in partial or complete vision loss. Conventional corneal transplant in these patients is associated with dismal results. Stem cell transplantation offers new hope to such patients. The umbilical cord is emerging as an important source of stem cells that may have potential clinical applications. There are advantages to the use of umbilical cord stem cells as these cells are less immunogenic, non-tumorigenic, highly proliferative and ethically acceptable. In this study,

we have confirmed the expression of several putative limbal stem cell markers such as HES1, ABCG2, BMI1, CK15 as well as cell adhesion-associated molecules INTEGRIN- α 6, - α 9, - β 1, COLLAGEN-IV and LAMININ in our recently characterized CLEC-muc population derived from human umbilical cord. Ex vivo expansion of these cells on a human amniotic membrane substrate formed a stratified cell sheet that similarly expresses some of these molecules as well as cornea-specific cytokeratins, CK3 and CK12. Transplantation of a bioengineered CLEC-muc sheet in limbal stem cell-deficient rabbit eyes resulted in regeneration of a smooth, clear corneal surface with phenotypic expression of the normal corneal-specific epithelial markers CK3, CK12 but not CK4 or CK1/10. Our results suggest that CLEC-muc is a novel stem cell that can be ex vivo expanded for corneal epithelial regeneration in the treatment of various eye diseases.

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Introduction

Corneal epithelium is a fast regenerating stratified tissue whose transparency is maintained by the integrity and functionality of a specialized stem cell population located at the basal region of the limbus, known as limbal stem cells [1–3]. Several diseases such as Stevens-Johnson syndrome, chemical burns, cicatricial pemphigoid, and aniridic keratopathy can result in limbal stem cell deficiency (LSCD) and cause devastating visual loss [3–7]. These conditions represent a major challenge for clinicians as conventional cornea transplantation is associated with dismal results. Transplantation of adult stem cells in the form of

autologous or allogeneic limbal stem cell has been used for treating these conditions with initial promising results [7–12]. More recently, cultured conjunctival transplantation has also been used to reconstruct the ocular surface to treat various eye diseases [13–15]. In many of these diseases where there is usually bilateral loss of limbal stem cells, transplantation of bioengineered oral mucosal epithelial cells has also been proposed [16, 17]. However, in majority of these cases, the grafted stem cells lose their proliferative capacity over time and these grafts eventually fail and repeat transplantation is often required [7].

Because of the enormous self-renewal potential and its ability to differentiate into multiple cell types, embryonic stem (ES) cells have generated tremendous interest to treat a vast range of diseases. However, there are major problems that have to be overcome before ES cell may be safely used for clinical applications because of its major ethical considerations, its uncontrolled differentiation, tumorigenesis, and risk of immunological rejection [18]. More recently, induced pluripotent stem cells (iPSC), which are derived by reprogramming of an adult fibroblast cell to form stem cells can be generated from a skin sample from an individual and this is generating a lot of interest as a potential autologous cell based therapy, because it eliminates the risk of immunological rejection and ethical issues [19, 20]. However, a risk-free cell therapy based on iPSC technology may still be many years away [21].

Umbilical cord is emerging as a novel potential source of stem cells which are highly proliferative. There are advantages to the use of umbilical cord stem cells as these cells are less immunogenic, non-tumorigenic, highly proliferative and ethically acceptable. Several studies have demonstrated the isolation and characterization of at least two types of cells: epithelial and mesenchymal from the cord [22–24]. These studies show that the umbilical cord epithelium expresses a cytokeratin pattern which is similar to human epidermis. Corneal epithelium and epidermis are derived from ectoderm during embryogenesis and these two tissues later differentiate to form non-keratinized and keratinized epithelia with different cytokeratin expression [25]. Furthermore, epithelial cells derived from umbilical cord are capable of forming a stratified epithelium when seeded on fibroblast populated collagen gels [23, 26]. Recently we have isolated and characterized a novel cell-type from human umbilical cord that expresses mucin1, hence termed as mucin-expressing cord lining epithelial cell (CLEC-muc) [27]. Our previous data demonstrate that CLECs-muc express several known ES cell markers including OCT-4, NANOG, SOX2, REX1 and SSEA-4 and also the putative limbal stem cell marker p63. We have shown that these cells are highly proliferative and can be induced to differentiate into CK3/CK12-positive cells [27]. In this study, we have further investigated the stem cell

characteristics of CLEC-muc and evaluated the use of CLEC-muc as an alternative to limbal stem cell to reconstruct and regenerate the corneal surface in a stem cell deficiency animal model.

We have found that CLECs-muc, in addition to p63, express other putative limbal stem cell markers ABCG2, HES1 and BMI1. We successfully engineered a stratified CLEC-muc cell sheet on human amniotic membrane (HAM), which expressed cornea-specific cytokeratins CK3 and CK12, INTEGRIN- β 1, COLLAGEN-IV, LAMININ but not CK4, CK13 or CK1/10. Transplantation of these cell sheets onto limbal stem cell-deficient rabbit eyes helped to regenerate a smooth and clear cornea epithelial surface. Molecular analysis of CLEC-muc grafted eyes revealed a gene expression profile that was similar to normal control eyes. Our study introduces a novel umbilical cord derived cell with high proliferative capacity which may have potential therapeutic applications for use in regenerative medicine and tissue replacement for treating various diseases.

Materials and Methods

Cell Culture

CLECs-muc were isolated from human cord lining tissue as described previously [27]. Briefly, surgical dissection of the umbilical cord was first performed to separate the umbilical cord lining membrane from the enclosed Wharton's jelly and other internal structures. The isolated cord lining membrane was then divided into small squares (0.5 cm²) for cell isolation. For CLEC-muc separation/cultivation, explant tissue samples were placed on cell culture plastic surfaces and submerged in 5 ml Medium-171 (Cascade Biologics, OR). Outgrowing cells were harvested by trypsinization (0.0125% trypsin/0.05% EDTA) in Medium-171 and cryopreserved. The cryopreserved CLECs-muc were thawed and cultured in PTTe-1 media supplemented with 2.5% fetal bovine serum (FBS), 50 μ g/ml insulin-like growth factor-1 (IGF-1), 50 μ g/ml platelet-derived growth factor-BB (PDGF-BB), 5 μ g/ml epidermal growth factor, 2 μ g/ml transforming growth factor- β 1 (TGF- β 1) and 5 μ g/ml insulin. We used cells from passage 5–8 from three donors for successive experiments.

Amniotic Membrane Preparation

Human amniotic membranes (HAMs) were obtained from mothers who had undergone cesarean sections after obtaining proper informed consent. Under sterile conditions, the membranes were washed with PBS that contained antibiotics (Amphotericin B, Penicillin G, Strptomycin and

Gentamicin) and were stored at -80°C in DMEM (Dulbecco's modified Eagle's medium; GibcoBRL, Rockville, MD) and glycerol (Wako Pure Chemical Industries, Osaka, Japan) in the ratio of 1:1 by volume. Immediately before use, the HAM was thawed, washed three times with sterile PBS that contained antibiotics, and cut into pieces approximately 4×4 cm in size. The overlying amniotic epithelial cells were removed by incubation with dispase (Gibco) at 37°C for 2 h, followed by gentle scraping with a cell scraper (Nunc International, Naperville, IL).

Development of a CLECs-muc Cell Sheet on an Amniotic Membrane Substrate

Cultured CLECs-muc were enzymatically separated using 1X trypsin-EDTA (Gibco). The separated cells were then seeded onto denuded amniotic membranes supported by nitrocellulose frame in 6-well culture plates, at a density of 1×10^5 cells/well. The cells were cultured in PTTe-1 media containing 10 ng/ml BMP4 [27] under submerged conditions for 7 days or 3 weeks. Cultures were incubated at 37°C in a 5% CO_2 -95% air incubator, and the media was changed every day.

Transplantation of CLEC-muc Sheet in a Limbal Stem Cell-deficient Rabbit Model

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To determine whether tissue-engineered CLEC-muc derived cell sheets were able to reconstruct the corneal surface, we developed an animal model of LSCD. Under general anesthesia, topical iodine-povidone 5% was instilled in the left eye preoperatively and we performed a complete anterior superficial keratectomy with removal of all the cornea epithelial cells and excision of the limbus. These eyes were divided into 3 treatment groups:

1. Group 1 (CLEC-muc-HAM group): eyes were transplanted with cultivated CLEC-muc cell sheets on HAM. The cultivated CLEC-muc HAM sheet was transplanted onto the denuded cornea stromal surface to completely cover the resected area. The sheet was sutured in place with 8 interrupted 10-0 nylon corneoscleral sutures.
2. Group 2 (HAM group): eyes were transplanted with plain denuded HAM alone.
3. Group 3 (No transplantation group): eyes were left bare.

After surgery, topical steroid (1% prednisolone acetate from Allergan, CA) was initially applied 1 drop four times a day and then tapered to three times a day over 4 weeks. The rabbit eyes were examined every week and photo-

graphed using a slit-lamp biomicroscope (Zoom Proto Slit Lamp, Nikon, Japan).

Histological Examination

10 weeks after surgery, the rabbits were sacrificed and the eyes were examined histologically. Samples were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 8 micron thickness and stained with hematoxylin-eosin.

Immunocytochemistry and Immunohistochemistry

Cultured cells on glass slide were fixed in cold methanol while the cultivated cell sheet and rabbit corneas were fixed in 4% paraformaldehyde and embedded in Tissue-Tek OCT compound for cryosectioning. Both samples were treated with 2% BSA in phosphate-buffered saline (PBS) with 0.4% Triton X-100 for blocking and permeabilizing. Primary antibodies were incubated overnight at 4°C in blocking buffer at 100X dilution and included mouse anti-CK1/10, goat anti-CK5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-CK3, mouse anti-HES1, rabbit anti-LAMININ (Millipore), mouse anti-CK19, mouse anti-ABCG2, mouse anti-CX43, mouse anti-CK4, mouse anti-INTEGRIN- $\beta 1$, mouse anti-INTEGRIN- $\beta 4$, mouse anti-INTEGRIN- $\alpha 6$, mouse anti-COLLAGEN-III, mouse anti-COLLAGEN-IV (Abcam, Cambridge, UK), rabbit anti-BMI1 (ProSci Incorporated) and mouse anti-KI67 (Sigma-Aldrich). Cells were stained with the appropriate fluorophore-conjugated secondary antibody (Invitrogen) at 500X dilution for 1 h at room temperature and counterstained with DAPI contained in the mounting media (Vectashield, Vector Laboratories, Burlingame, CA). Immunofluorescence images were examined using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany).

Semiquantitative and Quantitative Reverse Transcription-polymerase Chain Reaction

RNAs were obtained from cells using the RNeasy Mini kit (Qiagen), and cDNAs were generated using SuperScript III First Strand Kits (Invitrogen). For semiquantitative analysis, polymerase chain reaction (PCR) was conducted using PCR Master mix (2X) (Fermentas Life Sciences). Quantitative PCR was conducted in 25 μl of reaction mixture using LightCycler 480 and SYBR Green I Master kit (Roche Applied Science) under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 60°C for 50 s and elongation at 72°C for 30 s. All assays were run in duplicate for 3–5 individual samples. Relative expression levels were calculated using the ΔCt method, normalizing to glyceraldehyde-3-phosphate dehy-

drogenase (GAPDH) transcription. ΔCt was calculated by subtracting the Ct of GAPDH from the Ct of the target gene. For non comparison study, ΔCt values were graphed, therefore, higher Ct values indicate lower gene expression and vice versa. PCR products were verified by melting curve analysis and/or 2% agarose gel electrophoresis. The following oligonucleotide primer sequences were used:

Gene	Sequence	Gene	Sequence
<i>HES1</i>	For, 5'-GCG GAC ATT CTG GAA ATG ACA-3' Rev, 5'-AGC GCA GCC GTC ATC TG-3'	<i>INTEG-α6</i>	For, 5'-CAA GAT GGC TAC CCA GAT AT-3' Rev, 5'-CTG AAT CTG AGA GGG AAC CA-3'
<i>CK15</i>	zFor, 5'-GGA GGT GGA AGC CGA AGT AT-3' Rev, 5'-GAG AGG AGA CCA CCA TCG CC-3'	<i>INTEG-α9</i>	For, 5'-TGG ATC ATC GCC ATC AGT TTG-3' Rev, 5'-CCG GTT CTT CTC AGC TTC GAT-3'
<i>CK19</i>	For, 5'-CTG CGG GAC AAG ATT CTT GGT-3' Rev, 5'-CCA GAC GGG CAT TGT CGA T-3'	<i>SOX2</i>	For, 5'-GCA CAT GAA CGG CTG GAG AGC AACG-3' Rev, 5'-TGC TGC GAG TAG GAC ATG CTG TAG G-3'
<i>SOD2</i>	For, 5'-GCT GAC GGC TGC ATC TGT T-3' Rev, 5'-CCT GAT TTG GAC AAG CAG CAA-3'	<i>CK3</i>	Cat # PPH19500E (SABiosciences)
<i>CX43</i>	For, 5'-CCT TCT TGC TGA TCC AGT GGT AC-3' Rev, 5'-ACC AAG GAC ACC ACC AGC AT-3'	<i>Tp63</i>	Cat # PPH01032E (SABiosciences)
<i>GAPDH</i>	For, 5'-GCC AAG GTC ATC CAT GAC AAC-3' Rev, 5'-GTC CAC CAC CCT GTT GCT GTA-3'	<i>ABCG2</i>	Cat # PPH01526B (SABiosciences)
		<i>BMI1</i>	Cat # PPH5778A (SABiosciences)

Results

Gene Expression Profiling in Different Passages of CLECs-muc

A good primary cell strain must maintain not only the typical morphology but also the pattern of gene expression during passaging and thus confirming its quality. In our previous report, we demonstrated a novel stem cell population CLEC-muc from umbilical cord tissue [27].

We have further studied this cell line to understand the expression level of several genes found to be expressed in limbal/corneal epithelium by real-time RT-PCR when they were successively passaged. Total RNAs were collected from P5, P10 and P15 of CLECs-muc and Ct values were compared for genes including *ABCG2*, *BMI1*, *Tp63*, *HES1*, *SOD2*, *CK15*, *CK19*, *INTEGRIN- α 6*, *- α 9*, *SOX2*, *CX43* and *CK3*. Data revealed that CLECs-muc expressed putative stem cell as well as differentiation markers at varying quantities (Fig. 1), however, the expression levels did not change appreciably in different passages, suggesting that CLECs-muc retain their intrinsic cellular properties during passaging.

Expression of Putative Limbal Stem Cell Markers in CLEC-muc

We showed that CLECs-muc express p63, a putative stem cell marker for limbal stem cells [28, 29]. We further attempted to detect several other genes that have been suggested to be putative limbal stem cell markers such as *ABCG2*, *HES1* and *BMI1*. Real-time RT-PCR clearly identified these genes in RNA level (Figs. 1 and 3p). To confirm their expression at the protein level, we performed immunostaining using specific antibodies (Fig. 2a–o). Immunofluorescent staining showed that most of the CLECs-muc expressed *BMI1* (Fig. 2a–c) and *HES1* (Fig. 2d–f) in a fine-grain distribution pattern. *ABCG2* was also strongly detected in majority of the cells (Fig. 2g–i). A previous study demonstrates that *CK15* and Superoxide dismutase2 (*SOD2*) are expressed in clusters in limbal basal cells and thus have been proposed to be limbal stem cell and early transient amplifying cell markers in human adults [30]. When we examined their expression in CLEC-muc by RT-PCR, both *CK15* and *SOD2* transcripts were found in these cells (Figs. 1 and 3p). All these findings indicate that

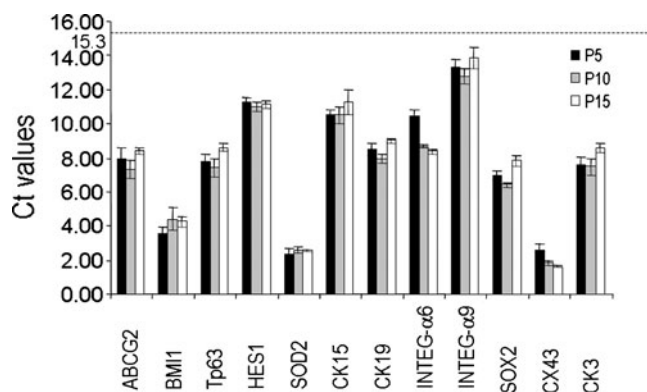


Fig. 1 CLEC-muc expresses putative limbal stem cell markers. Expression of *ABCG2*, *BMI1*, *Tp63*, *HES1*, *SOD2*, *CK15*, *CK19*, *INTEGRIN- α 6*, *INTEGRIN- α 9* and *SOX2* was maintained in CLEC-muc over the passages. Notably, differentiation marker *CX43* and *CK3* are also expressed at significant levels

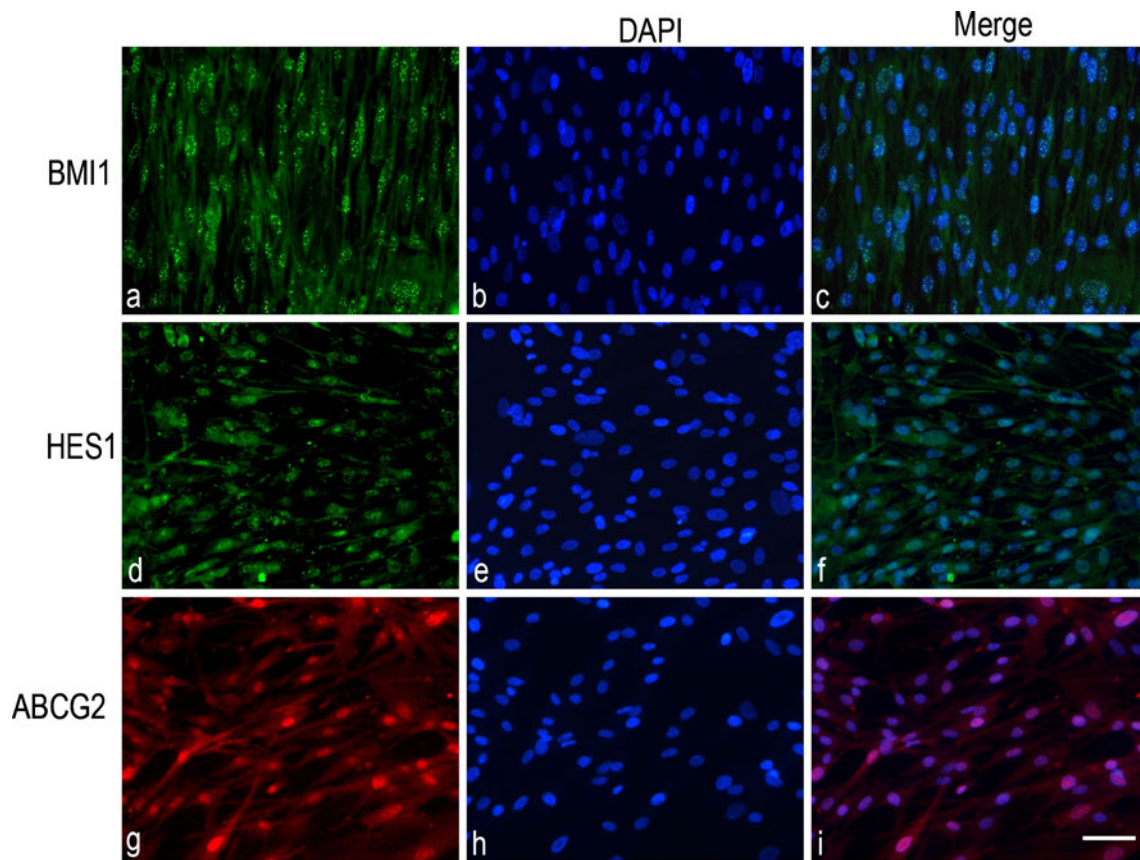


Fig. 2 Cellular localization of limbal stem cell markers by fluorescent immunostaining. CLECs-muc were cultured for 7 days and subjected to immunostaining against BMI1 (**a**), HES1 (**d**) and ABCG2 (**g**).

DAPI depicts cell nuclei (**b**, **e**, **h**). Merged images are shown in **c**, **f** and **i**. Each image is the representative of at least three independent experiments. Bar, 50 μ m

CLECs-muc possesses adult epithelial stem cell properties, and could possibly differentiate into a corneal phenotype.

Expression of Cell Adhesion and Related Molecules

Integrins represent one of the important classes of cell adhesion molecules. Limbal and corneal basal cells have been found to exhibit differential expression patterns for INTEGRIN- β 1, - β 4, - α 6 and - α 9 [31]. We profiled the expression of these molecules in CLECs-muc by immunostaining and RT-PCR. Although INTEGRIN- β 1 was strongly expressed (Fig. 3a–c), INTEGRIN- β 4 and - α 6 were not detected by immunostaining. However, RT-PCR exhibited positive expression of INTEGRIN- α 6 and - α 9 (Fig. 1p). We also examined the expression of basement membrane proteins, LAMININ and COLLAGEN type IV, and found that these molecules were strongly expressed in CLECs-muc (Fig. 3d–f, j–l). A corneal stromal matrix protein, COLLAGEN type III [32] was also examined in our cells and this was found to be positive by immunostaining (Fig. 3g–i). Finally, we examined the expression of the gap junction protein CX43 [33]. Both immunostaining and RT-PCR revealed the presence of this molecule in

CLEC-muc (Fig. 3m–p). This data indicate that CLECs-muc share some common characteristics with limbal/corneal cells.

Gene Expression Analysis and Characterization of Cultivated Cell Sheet

CLECs-muc were cultivated on denuded HAM substrate to form confluent cell sheets and then we evaluated the expression of tissue-specific cytokeratins, gap-junction proteins and basement membrane proteins in these sheets. Immunodetection revealed a positive expression of cornea-specific differentiation cytokeratins CK3 and CK12 [34] (Fig. 4a, b and c, d, respectively). Expression of the conjunctival epithelial marker, CK4 [35], was undetectable in the tissue construct (Fig. 4e, f). The putative stem cell marker HES1 and proliferation marker KI67 were also detected (Fig. 4g, h and i, j, respectively). CX43, a well-known gap junction protein expressed in differentiated cells, was found throughout the cell sheet (Fig. 4k, l). Similarly, the membrane assembly proteins, INTEGRIN- β 1 (Fig. 4m, n), COLLAGEN-IV (Fig. 4o, p) and LAMININ (Fig. 4q, r) were expressed, while INTEGRIN- α 6 and - β 4 were not observed.

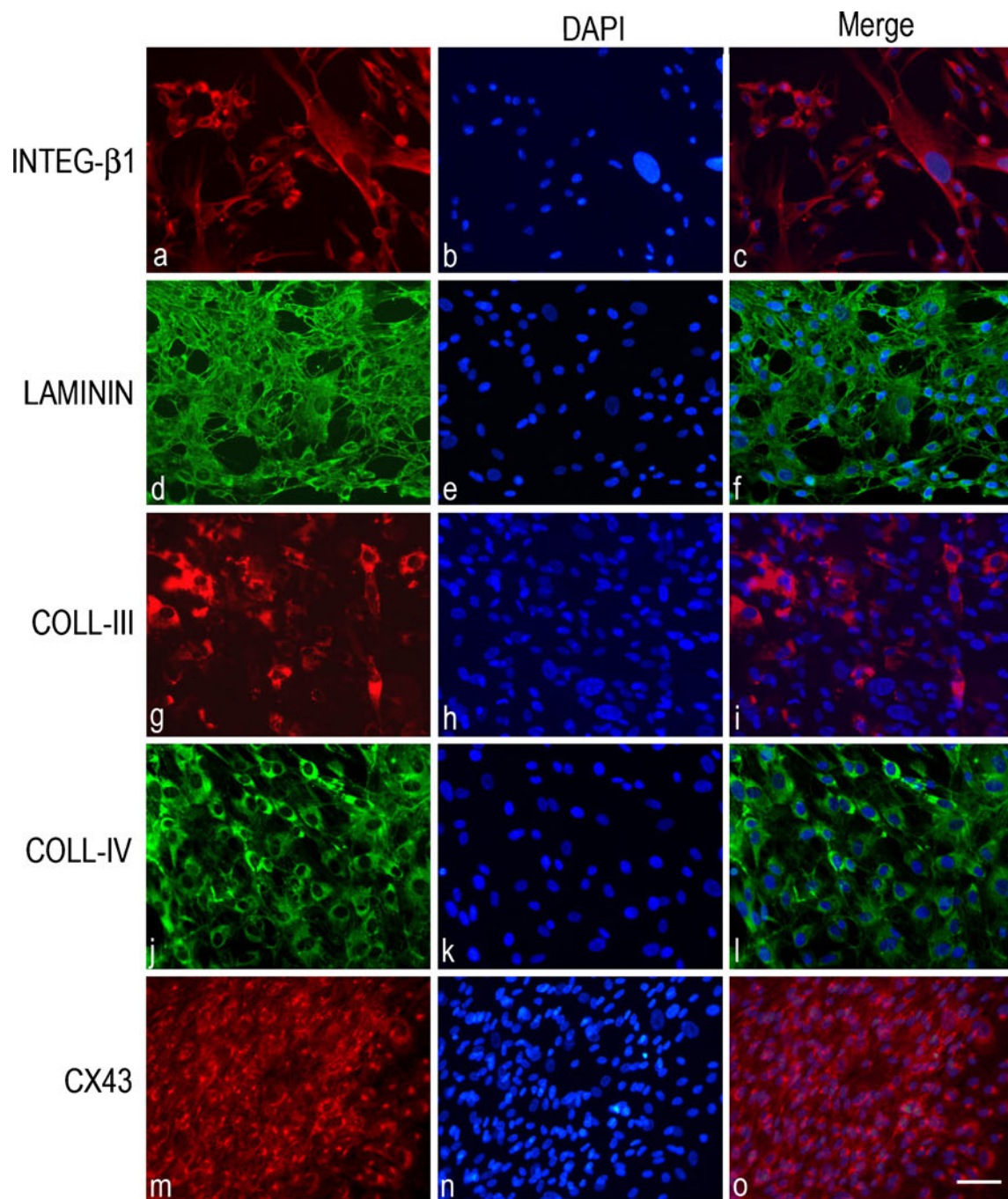
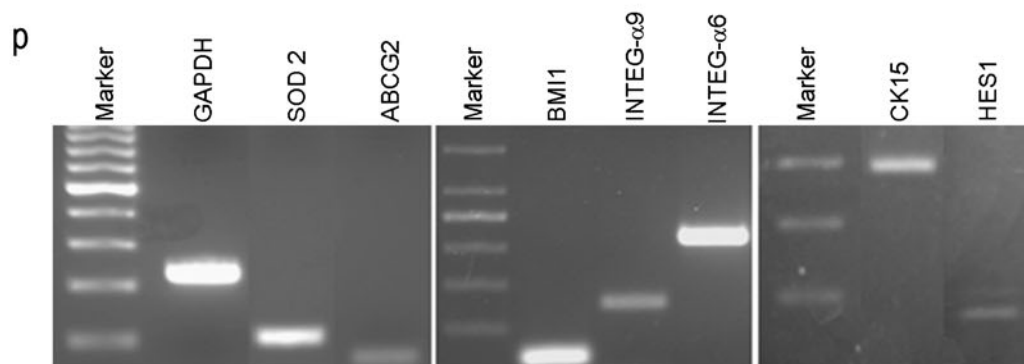


Fig. 3 Expression of cell adhesion-related molecules was observed in CLEC-muc. Cultured CLECs-muc subjected to immunostaining against specific antibodies exhibited positive expression for INTEGRIN- β 1 (a), LAMININ (d), COLLAGEN-III (g), COLLAGEN-IV (j) and CX43 (m). DAPI depicts cell nuclei (b, e, h, k, n). Merged images are shown in c, f, i, l, o. Each image is the representative of at least three independent experiments. Bar, 50 μ m. RT-PCR using total RNA from cells cultured in same condition also showed the expression of putative limbal stem cell markers SOD2, ABCG2, BMI1, INTEGRIN- α 9, INTEGRIN- α 6, CK15 and HES1 (p)

Transplantation of CLEC-muc Sheet onto LSCD Rabbit Eyes

Examination of the rabbit corneas up to 4 weeks after surgery revealed differences in the clinical appearance between the 3 treatment groups (Fig. 5a–c). Eight eyes underwent CLEC-muc-HAM transplantation, of which two eyes were excluded from the analysis because the grafts dropped off within a few days of the transplant. Of the 6 eyes that retained the graft, 4 eyes exhibited a clear and smooth cornea surface with minimal corneal neovascularization or opacification (Fig. 5a), 1 eye had a clear surface with 3 clock hours of corneal neovascularisation and 1 eye had mild superficial haziness but the surface remained

intact and there was minimal neovascularization. In contrast, all 4 eyes in the HAM transplant group showed severe signs of LSCD (Fig. 5b) with cornea epithelial defects, irregularity of the surface, corneal opacification, and superficial and deep corneal neovascularization. In the eyes with no transplant, all 4 eyes also exhibited signs of LSCD (Fig. 5c). Ten weeks after surgery, the corneas remained clear in the CLEC-muc-HAM group (Fig. 5d). In the groups that had received only HAM transplants or no transplants, the cornea remained hazy and there were persistent epithelial defects and irregularity of the corneal surface, cornea opacification, and superficial and deep corneal neovascularization (Fig. 5e, f).

Histological examination of the eyes revealed that the CLEC-muc-HAM transplanted group formed a well laminated 5–7 layer stratified epithelium with a smooth surface (Fig. 5g), which was similar in appearance to normal in vivo corneal epithelium (Fig. 5h). In contrast, in the group that was not transplanted, a thick, slightly irregular epithelium consisting of 7–9 layers of cells was observed (Fig. 5i). For the HAM transplanted group, a relatively thin epithelium with multiple infiltrating inflammatory cells beneath the basal layer was noted (Fig. 5j).

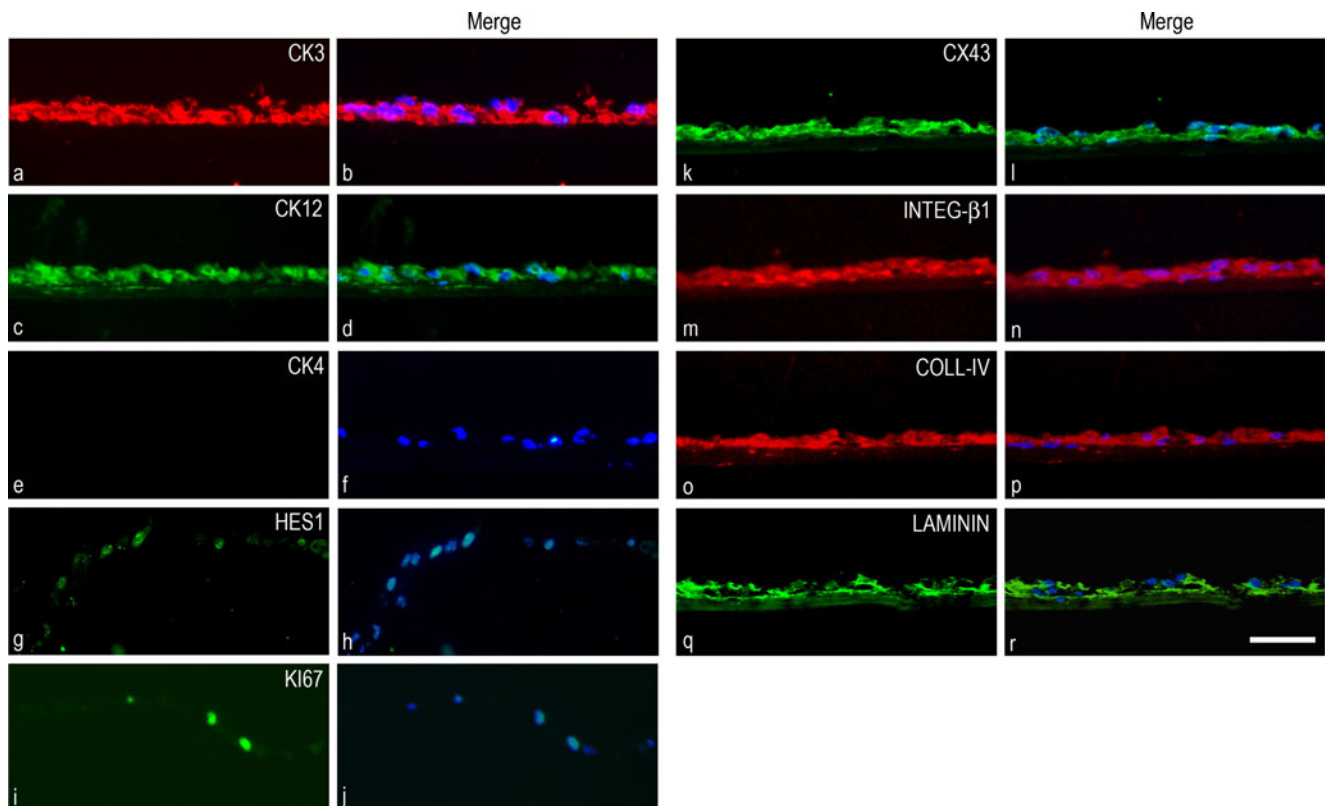


Fig. 4 Immunohistochemical detection of protein expression in engineered CLEC-muc sheet. Corneal epithelial markers CK3 (a) and CK12 (c) were expressed however, conjunctival marker CK4 was not detected (e). HES1 (g) and KI67 (h) were observed in limited number of cells. Adhesion-related molecules CX43 (k), INTEGRIN- β 1 (m),

COLLAGEN-IV (o) and LAMININ (q) were also expressed in this sheet. DAPI shows cell nuclei in merged images (b, d, f, h, j, l, n, p, r). Each image is the representative of at least three independent experiments. Bar, 100 μ m

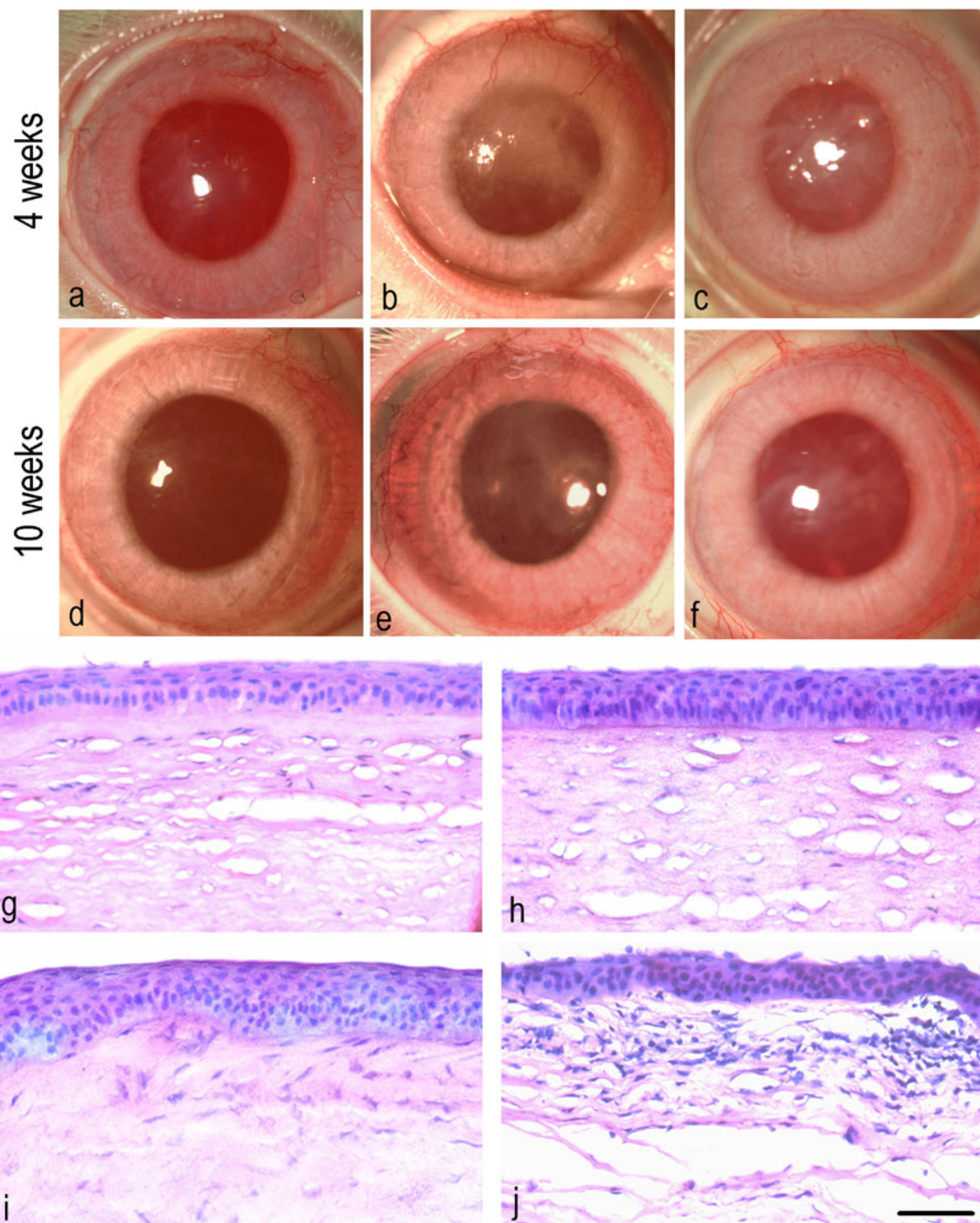


Fig. 5 Representative images show the gross appearances after transplantation of CLEC-muc-HAM sheet to the damaged corneal surface of rabbit. CLEC-muc-HAM transplanted sheet displayed very little opacity after 4 weeks (**a**), which almost disappeared after 10 weeks (**d**). HAM transplanted eyes showed significant opacity in cornea after 4 weeks, which reduced but remained after 10 weeks (**b**, **e**, respectively). No transplant eyes exhibited opacity and neovascularization in cornea after 4 (**c**) and 10 weeks (**f**). Sections of these eyes

upon hematoxylin-eosin staining revealed almost regular smooth corneal epithelium with no infiltrating cells in CLEC-muc-HAM group (**g**); while a thick corneal epithelium with some infiltrating cells in stroma in no transplant group (**i**); and irregular corneal epithelium with many infiltrating cells in stroma in HAM transplanted group (**j**). Figure **h** shows normal structure of un-treated control corneal epithelium. Bar, 100 μ m

Immunocharacterization of CLEC-muc Grafted Eyes

CK3 was expressed throughout the cornea epithelial surface in the CLEC-muc-HAM group (Fig. 6a, b), which was similar to that of the intact normal cornea control eye (Fig. 6g, h). CK3 expression in the eyes that had plain HAM transplantation or no transplantation was significantly reduced and was discontinuous (Fig. 6c, d and e, f, respectively). When we examined CK12 expression, CK12 was only detected in the CLEC-muc-HAM group. Its expression was mainly in the superficial layers as compared to normal untreated control epithelium (Fig. 6i, j and o, p, respectively). This probably corresponds to the differentiated cells located superficially and the less differentiated cells located in the basal region during the period of follow-up. In contrast, CK12 expression was absent in the eyes that had undergone only HAM transplantation or had no transplants (Fig. 6k, l and m, n, respectively). We also checked the expression of conjunctival markers CK4 and CK19 in these eyes. Immunostaining study showed no expression of CK4 (Fig. 7a, b) and CK19 (Fig. 7i, j) in CLEC-muc-HAM transplanted eyes, suggesting that these eyes were resurfaced by cells possessing a cornea-like phenotype. This was similar to that of the intact normal cornea control eye (Fig. 7g, h and o, p, respectively). In contrast, there was strong expression of CK4 and CK19 in the eyes with no transplantation (Fig. 7e, f and m, n, respectively) and moderate expression of CK4 and CK19 in the HAM group (Fig. 7c, d and k, l, respectively) suggesting that the corneal surface in these eyes were repopulated by conjunctival epithelium (conjunctivalization) rather than normal corneal epithelium. These findings suggest that bioengineered CLEC-muc cell sheets were able to regenerate the corneal surface with cornea-like epithelium bearing the morphological and phenotypic characteristics of normal cornea epithelium.

Discussion

CLECs-muc Possess Limbal/Corneal Cell Specific Features

Stem cells have the ability to form different cell types being exposed to specific extrinsic factors in vitro and in vivo [35]. The intrinsic cell property plays a decisive role in fate determination. We previously described the isolation and characterization of this novel stem cell population from human umbilical cord and showed that it possessed both embryonic stem cell markers as well as an adult limbal stem cell marker, p63 [27]. In this study, we demonstrated that CLECs-muc expressed several other limbal stem cell markers including HES1, BMI1 and ABCG2 (Figs. 1 and 2). In addition, CLEC-muc expressed CK15 and SOD2 [30] that are considered to be expressed in limbal stem/progenitor cells

along with p75 [36], an oral epithelial stem/progenitor cell marker. These findings suggest that CLECs-muc share some common stem cell characteristics with limbal stem cells and may be able to differentiate towards a corneal epithelial phenotype, and therefore, could potentially help to regenerate the cornea epithelium in vivo.

Cell adhesion molecules are important mediators of cell-to-cell interaction. These molecules also interact with extracellular matrix proteins for anchoring, communication and signaling. We observed that CLECs-muc expressed cell adhesion molecule INTEGRIN- β 1, gap junction protein CX43, extracellular matrix molecule COLLAGEN type III, and basement membrane proteins LAMININ and COLLAGEN type IV (Fig. 3). These findings suggest that CLECs-muc maintain cell-to-cell contact and interaction and are able to attach to the basement scaffold when they are bioengineered to form a cell sheet. These cell adhesion molecules were also present in the ex vivo expanded cell sheet. The ability of these cells to remain attached to the substrate during and following transplantation is critical when considering the use of these cells for clinical applications.

Ex-vivo Expansion of CLECs-muc on HAM

We succeeded in generating a stratified multilayered cell sheet following 2–3 weeks of ex vivo expansion of CLEC-muc on denuded HAM (data not shown). HAM is at present the substrate of choice for cultivating cells for cornea and ocular surface transplantation, because of its ability to maintain the undifferentiated state of cells, and these cultivated epithelial-HAM transplants have been used clinically for the treatment of various eye diseases [11–15, 37–39]. Our results showed that the corneal differentiation marker CK3 was strongly expressed while the conjunctival cytokeratin CK4 was totally absent in our cell sheet (Fig. 4c, e). Gap junction protein CX43 and adhesion molecule INTEGRIN- β 1 were also expressed. On the other hand, the proliferation marker KI67, and putative stem cell marker HES1 were observed only in a few number of cells (Fig. 4g, i) while epidermal keratinization marker CK1/10 was not detected [40]. We demonstrated that CLEC-muc can differentiate into a non-keratinized corneal epithelial cell-type that specifically expresses CK3/12 but not CK1/10.

Transplantation of Tissue-engineered CLEC-muc-sheet Reconstructs Corneal Epithelium

Recognizing the key role of stem cell niche in stem cell fate and differentiation [41], we finally performed in vivo experiments to assess the feasibility of using tissue-engineered cell sheets to reconstruct corneal epithelium in LSC-deficient rabbits. We hypothesized that the microen-

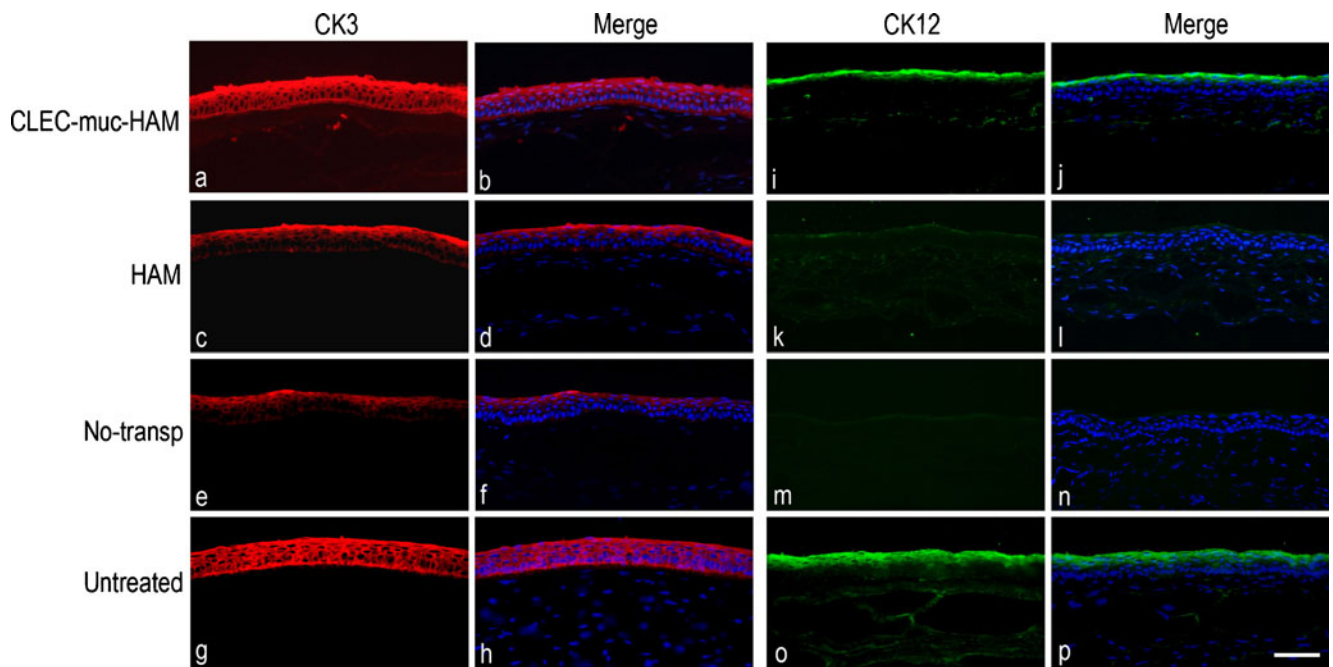


Fig. 6 Corneal epithelial differentiation markers CK3 and CK12 were expressed in reconstructed rabbit cornea after CLEC-muc-HAM transplantation. CLEC-muc-HAM exhibited strong expression of CK3 as comparable to that of untreated control cornea (a, g, respectively), however, a less and discontinuous expression was found in HAM and no transplant groups (c, e, respectively). CK12-positivity

was only detected in CLEC-muc-HAM group (i), although this was less than that of untreated control cornea (o). HAM transplanted and no transplant groups did not express CK12 (k, m, respectively). Blue DAPI visualizes cell nuclei in merged images (b, d, f, h, j, l, n, p). Each image is the representative of at least three independent experiments. Bar, 100 μ m

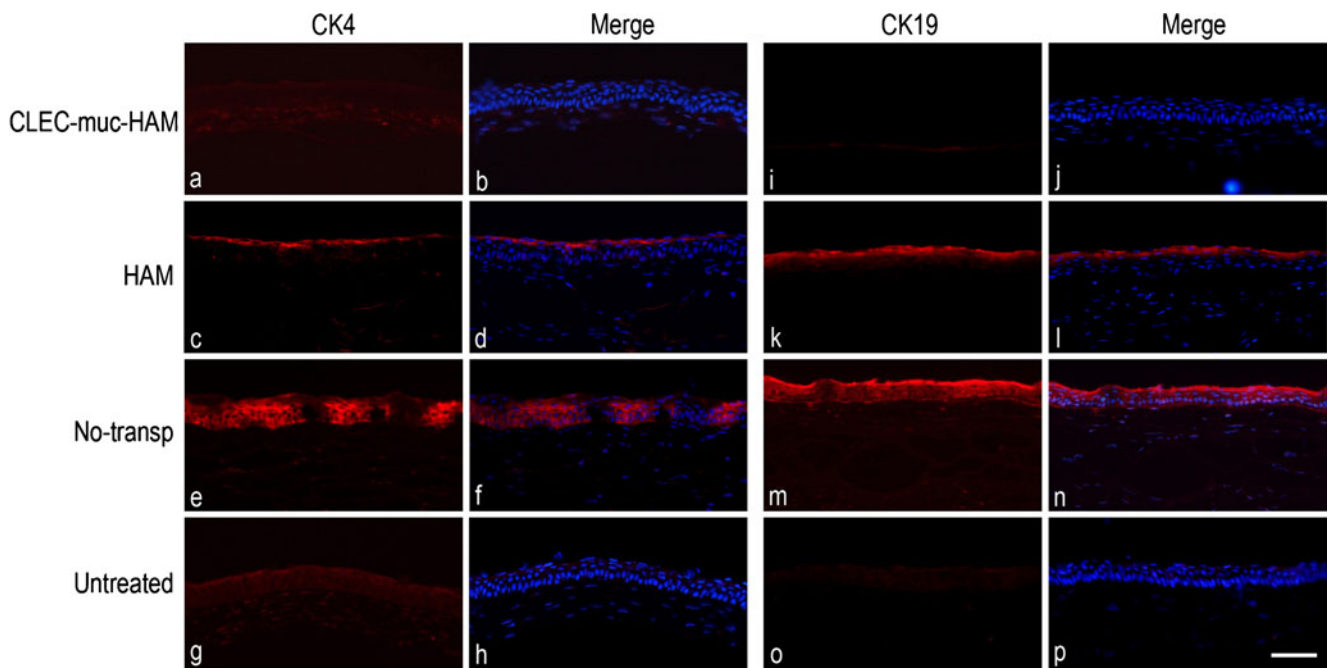


Fig. 7 Conjunctival epithelial markers CK4 and CK19 were absent in reconstructed rabbit cornea after CLEC-muc-HAM transplantation. CLEC-muc-HAM showed no expression of CK4 as was found in untreated control cornea (a, g, respectively), however, a less expression was observed in HAM transplanted group (c). A relatively strong but discontinuous expression was detected in no transplant group (e). Similarly, CK19 was not detectable in CLEC-muc-HAM

group (i), as was found untreated control cornea (o). HAM transplanted group exhibited positive expression only in superficial layers (k) while no transplant group expressed stronger expression of CK19 (m). Blue DAPI visualizes cell nuclei in merged images (b, d, f, h, j, l, n, p). Each image is the representative of at least three independent experiments. Bar, 100 μ m

vironment of the cornea and limbal region may help to direct CLEC-muc towards a corneal cell. Our study shows that tissue-engineered cell sheets derived from CLECs-muc were able to successfully regenerate the damaged cornea surface in LSC-deficient eyes (Fig. 5a, d, g) with a cornea-like epithelium that was clear and smooth with minimal associated corneal neovascularization. Histological and immunohistochemical examination revealed that the cellular organization in these CLEC-muc-HAM transplanted eyes resembled that of normal healthy cornea epithelium. On the other hand, an irregular surface with many infiltrating cells in the corneal stroma was apparent in HAM transplant group. The eyes without transplantation were re-epithelialized by conjunctival epithelium (conjunctivalization). The CLEC-muc-HAM transplant group demonstrated the normal corneal phenotype with CK3 and CK12 expression and absence of expression of conjunctival markers CK4 and CK19 (Figs. 6 and 7). In contrast, in both other treatment groups, the corneal surfaces were repopulated by conjunctival epithelium which expressed conjunctival markers CK4 and CK19. These results suggest that this new source of cells may be an effective method for reconstructing the corneal epithelium.

The success of allogeneic cell transplantation largely depends on the degree of immunogenicity of the donor cell, an intrinsic cell property, as well as how immunoprivileged is the graft site, a property of the recipient [42]. Immunoprivileged corneal epithelial cells strongly express MHC class I but not MHC class II antigens [43]. We have similarly found that CLECs-muc are negative to MHC class II expression [27]. More recently an *in vivo* study clearly shows that CLECs-muc when injected in mice kidney capsules do not form teratomas and inflammation was minimal, suggesting that CLEC-muc is non-immunogenic and non-tumorigenic [44]. Our study showed that there was minimal neovascularization, inflammation and no graft rejection during the follow-up period when the rabbits received allogeneic CLECs-muc transplants despite being xenografts without the use of immunosuppression. The short-term results of transplanting these cells are promising. As such, there are advantages to the use of umbilical cord stem cells compared to ES cells as these cells are less immunogenic, non-tumorigenic, highly proliferative and ethically acceptable. Long-term studies will be carried out to determine the long-term outcome of these transplants.

Transplantation of CLEC-muc may prove especially useful for patients with bilateral LSCD, for whom transplantation of autologous limbal stem cells is not possible. Recent progress in this field suggests that autologous oral mucosal cultured epithelial cells [17, 45, 46] and conjunctival epithelial cells [47] may serve as alternative options for treating these severe ocular surface diseases. However there are limitations with the use of adult stem cells, which

include its (a) limited proliferation ability, which is why these transplants often fail in the long-term and repeat surgery may be required; (b) the very low number of cells in adult tissues; (c) the difficulty in culturing cells outside the body and (d) the risk of graft rejection with allografts.

CLEC-muc has advantages over adult stem cells, ES cell and iPSC. It is easily available, has no ethical issues related to its use, is highly proliferative, is closer to adult stem cells compared to ES cell and iPSC, is non-tumorigenic with no teratoma formation, is less immunogenic with less risk of graft rejection, and can be cryopreserved as a readily available source of stem cells for clinical use.

In conclusion, our study demonstrates that CLECs-muc express putative limbal stem cell markers p63, ABCG2, HES1, BMI1, CK19 and CK15 and, these cells can be *ex vivo* expanded on HAM to generate cell sheets. The cultured cells are immunologically naïve and phenotypically similar to corneal epithelial cells that express corneal differentiation marker CK3/12 but not CK4 or CK10. We have shown that transplantation of CLECs-muc effectively regenerates the cornea surface with cornea-like epithelium. As umbilical cord is easily obtainable and there are no ethical concerns related to its use, CLEC-muc may serve as a readily available source of stem cells that may prove to be a better and safer stem cell source. These novel stem cells have potential important clinical applications for use in regenerative medicine and tissue replacement for treating various diseases.

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