Human pluripotent stem cell-derived limbal epithelial stem cells on bioengineered matrices for corneal reconstruction

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Abstract
Conical epithelium is renewed by limbal epithelial stem cells (LESCs), a type of tissue-specific stem cells located in the limbal palisades of Vogt at the corneo-scleral junction. Acute trauma or inflammatory disorders of the ocular surface can destroy these stem cells, leading to limbal stem cell deficiency (LSCD) — a painful and vision-threatening condition. Treating these disorders is often challenging and complex, especially in bilateral cases with extensive damage. Human pluripotent stem cells (hPSCs) provide new opportunities for corneal reconstruction using cell-based therapy. Here, we investigated the use of hPSC-derived LESC-like cells on bioengineered collagen matrices in serum-free conditions, aiming for clinical applications to reconstruct the corneal epithelium and partially replace the damaged stroma. Differentiation of hPSCs towards LESC-like cells was directed using small-molecule induction followed by maturation in corneal epithelium culture medium. After four to five weeks of culture, differentiated cells were seeded onto bioengineered matrices fabricated as transparent membranes of uniform thickness, using medical-grade porcine collagen type I and a hybrid cross-linking technology. The bioengineered matrices were fully transparent, with high water content and swelling capacity, and parallel lamellar microstructure. Cell proliferation of hPSC-LESCs was significantly higher on bioengineered matrices than on collagen-coated control wells after two weeks of culture, and LESC markers p63 and cytokeratin 15, along with proliferation marker Ki67 were expressed even after 30 days in culture. Overall, hPSC-LESCs retained their capacity to self-renew and proliferate, but were also able to terminally differentiate upon stimulation, as suggested by protein expression of cytokeratins 3 and 12. We propose the use of bioengineered collagen matrices as carriers for the clinically-relevant hPSC-derived LESC-like cells, as a novel tissue engineering approach for corneal reconstruction.

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1. Introduction

Corneal clarity is essential for normal vision and is largely dependent on stromal structure, avascularity, and epithelial integrity. Corneal epithelium, the outermost layer of the transparent and avascular cornea, is stratified and rapidly regenerating.
It is renewed by limbal epithelial stem cells (LESCs), a type of tissue-specific stem cells located at the corneo-scleral junction within the niche regions of the palisades of Vogt (Di Girolamo 2011, Dua and Azuara-Blanco 2000). LESC also act as a barrier between the conjunctiva and the cornea, preventing conjunctival epithelial cells from migrating to the corneal epithelium. When LESC are damaged as a result of severe trauma or disease, corneal epithelial renewal is disrupted and the neighboring conjunctival epithelial cells migrate over the corneal surface (Ahmad 2012, Pellegrini et al., 2014). These types of ocular surface disorders are collectively referred to as limbal stem cell deficiency (LSCD), and they vary in their severity depending on whether the damage is unilateral or bilateral, partial or total. Corneal transplantation alone is not a feasible option for patients suffering from LSCD, as the corneal grafts only replace the central cornea, and not the limbus, thereby relying on the patient’s own LESC to continuously regenerate the corneal epithelium (Ahmad et al., 2010). Various alternative approaches have been proposed to facilitate the reconstruction of damaged ocular surface. These include transplantation of autologous limbus from the healthy eye in unilateral cases, or allogeneic limbal tissue from living or cadaveric donors when both eyes are affected (Dua and Azuara-Blanco 2000). More recently, cultivated limbal epithelial transplantation (CLET) using either autologous (dry) or allogeneic LESC has been introduced (Pellegrini et al., 1997; Rama et al., 2010; Ramachandran et al., 2014). The overall success rate of this technique is around 76%, varying greatly due to differences in study set-ups (Baylis et al., 2011). Other limitations of CLET, besides variation in long-term success rates, include the use of xenogeneic and undefined culture components, and scarcity of healthy limbal donor tissue. Furthermore, the patient’s vision often remains poor due to stromal scarring, making corneal transplantation necessary once the limbus has been stabilized (Joe and Yeung, 2014). Shortage of donor tissue is especially limiting in the case of total bilateral LSCD, making it necessary to explore other cell sources besides LESC, such as oral mucosal epithelium or hair follicle stem cells (Blazejewska et al., 2009; Nishida et al., 2004). Human pluripotent stem cells (hPSCs), namely human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provide new opportunities for cell-based tissue engineering, as they possess excellent self-renewal qualities and are therefore readily available in limitless supply. Moreover, hiPSC-derived cells offer novel ways to study human development, and discover and test new drugs. We have previously described a method for differentiating corneal epithelial progenitor cells from hiPSCs under feeder-free and serum-free conditions (Mikhailova et al., 2014). Robust differentiation of LESC-like progenitor cells along with elimination of undefined components makes the resulting cell populations clinically relevant, as there is a smaller risk of pathogen transfer or batch-to-batch variation linked to the use of feeder cells and serum.

Finding a scaffold suitable for transplantation of cells to the ocular surface is an important step towards clinical applications. Ideally, the scaffold should be transparent, mechanically robust and sufficiently elastic to withstand manipulation and suturing, while supporting cell growth. Human amniotic membrane (hAM) is the most extensively studied substrate for culture and transplantation, although its natural biological and thickness variability, as well as poor standardization, cause significant variation in clinical outcomes (Allen et al., 2013; Shortt et al., 2009). Several studies have addressed this issue, proposing various biomaterials for ocular surface therapy using LESC, such as fibrin, keratin, silk fibroin and collagen (Feng et al., 2014; Liu et al., 2012; Petsch et al., 2014; Rama et al., 2010). Bioengineered corneal implants fabricated using collagen, the principal building block of corneal stroma, have shown promise in preclinical studies (Li et al., 2003). Most recently, we reported successful implantation of cell-free bioengineered matrices fabricated from medical-grade collagen type I (Koulikovska et al., 2015). The matrix was tested in a rabbit model in a new type of intrastromal surgery for its therapeutic potential in regenerating the corneal stroma. The bioengineered matrix was robust with good tunable physical and optical properties, and when implanted, it integrated rapidly into corneal structures with minimal signs of inflammation or damage to the surrounding tissue, demonstrating that the material is biocompatible despite the collagen being of xenogenic origin. Host stromal cells migrated into the implant matrix and either remained quiescent or initiated regeneration (Koulikovska et al., 2015). Here, we propose the use of such bioengineered medical-grade collagen matrices as potential therapeutic carriers for hiPSC-derived LESC-like cells (hPSC-LESCs), aiming to reconstruct the corneal epithelium and partially replace the underlying stroma. The present study was carried out in serum-free culture conditions, minimizing undefined components to yield more reproducible and clinically relevant results. To our knowledge, this is the first study evaluating the proliferation and differentiation of hPSC-LESCs cultured on standardized biomaterial scaffolds in serum-free conditions.

2. Materials and methods

2.1. Fabrication of bioengineered matrices

Bioengineered matrices were developed by hybrid cross-linking of reconstituted collagen molecules. A hybrid mixture of 1-3-(dimethylamino)propyl-3-ethylcarbodiimide methiodide (EDCM; Sigma-Aldrich-St. Louis, USA) and dicyclohexyl-carbodiimide (DCC; Thermo Fisher Scientific, Rockford, IL USA) were used for cross-linking and polymerization of a medical grade, high purity collagen (type I atelo-collagen) extracted from porcine skin. High collagen content (18%) was achieved by a controlled vacuum evaporation of a dilute solution (5%) of collagen at room temperature. The vacuum evaporation method helped to increase the collagen concentration without compromising transparency of the matrices. EDCM was dissolved in sterile water, and DCC was dissolved in 70% ethanol, forming 20% solutions. Both cross-linking agents were added to the 18% collagen solution at molar ratios (0.5:0.5:1; EDCM: DCC: collagen), mixed thoroughly and molded in between glass plates to make a homogeneous bioengineered matrix. A 100 μm thick spacer and a clamping system were used for compression molding of the matrices. Samples were then cured at room temperature in 100% humidity chambers for 25 h in order to complete the cross-linking reaction. De-molding was achieved by immersion in phosphate buffered saline (PBS) for 1 h. Samples were subsequently washed four times with 1 × PBS containing 1% chloroform to extract reaction byproducts, and to sanitize the samples.

2.2. Water content and swelling capacity of bioengineered matrices

Water content and swelling capacity studies were performed for matrices and donor human corneas that were equilibrated in 1 M PBS solution. Five replicate samples were used for this test. The research grade human donor corneas were obtained from the Eye Bank of Canada. Briefly, samples were lightly touched with a clean Kimwipe cloth to remove the surface water and weighed immediately (hydrated mass). The samples were then placed in separate petri dishes, air-dried for 6 h and weighed again (dry mass). Equilibrium hydrated mass \( m_{\text{hydrated}} \) and dry mass \( m_{\text{dry}} \) were used to determine water content and water uptake, defined as follows:

\[
\text{Water content} = \frac{m_{\text{hydrated}} - m_{\text{dry}}}{m_{\text{dry}}} 
\]

\[
\text{Water uptake} = \frac{m_{\text{hydrated}} - m_{\text{dry}}}{m_{\text{dry}}} \times 100\%
\]
Water content = \( \left( \frac{m_{\text{hydrated}} - m_{\text{dry}}}{m_{\text{hydrated}}} \right) \times 100\% \).

Water uptake = \( \left( \frac{m_{\text{hydrated}} - m_{\text{dry}}}{m_{\text{dry}}} \right) \times 100\% \).

### 2.3. Optical properties of bioengineered matrices

Light transmission and scatter measurements of the bioengineered matrix were carried out at room temperature for white light and for narrow spectral regions (centered at 450 nm, 550 nm, and 650 nm), using a custom-built optical instrument, as previously described (Koulikovska et al., 2015). Samples were hydrated in PBS before and during the measurement. The light transmission values for healthy human corneas were adopted from the literature.

### 2.4. Microstructure of bioengineered matrices

Scanning electron microscopy (SEM) was performed using a Jeol JSM 6320F microscope. The bioengineered matrices that were stored and equilibrated in PBS were frozen for 16 h, followed by lyophilization for 24 h. Samples were sputtered with a 2 nm gold coating to allow for conductivity prior to mounting for SEM. Samples were then examined using an accelerating voltage of 10 kV.

### 2.5. Human pluripotent stem cell lines

Two hPSC lines were used in this study. The hESC line (Regea08/017, 46XX) was previously derived at the University of Tampere (Skottman 2010). The hiPSC line (UTA.04511.WT, 46XY) was reprogrammed from human dermal fibroblasts using the CytoTune®-iPS Sendai Reprogramming Kit (Invitrogen, Carlsbad, CA) by Katriina Aalto-Setälä’s group at the University of Tampere (Ojala et al., in press). Both cell lines were routinely maintained on mitotically inactivated human foreskin fibroblast (CRL-2429, ATCC, Manassas, VA) feeder cells in serum-free conditions, and enzymatically passaged onto fresh feeder cell layers at ten-day intervals. To confirm their quality, both cell lines were routinely characterized roughly every 20 passages. Protein expression of several pluripotency markers was verified using immunofluorescence (described in Chapter 2.7), and karyotyping analysis was performed by the Finnish Microarray and Sequencing Center using high-throughput KaryoLITE™ BoBSTM assay (Lund et al., 2012). For in vitro assay of pluripotency, undifferentiated hPSC colonies were mechanically dissected to obtain embryoid bodies, which were formed and cultured in the absence of basic fibroblast growth factor (bFGF). The first two weeks of culture were carried out in suspension, followed by one week on well-plates coated with recombiant human vitronectin (0.68 μg/cm²; Thermo Fisher Scientific). The formation of mesodermal, endodermal and ectodermal derivatives was verified using immunofluorescence.

### 2.6. Human PSC-LESC differentiation and culture on bioengineered matrices

Differentiation of hPSCs towards LESC-like cells was carried out as previously described (Mikhailova et al., 2015). Briefly, differentiation was first induced towards surface ectoderm in suspension culture in the presence of the transforming growth factor β (TGF-β) inhibitor SB-505124, Wnt inhibitor IWP-2 (both from Sigma–Aldrich), and bFGF (PeproTech, Rocky Hill, NJ). Thereafter, cell aggregates were plated onto 12-well plates (Corning CellBIND; Corning, NY) coated with 5 μg/cm² human placental collagen IV (Sigma–Aldrich), and maintained in a defined and serum-free medium CnT-30 (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) developed for differentiation of primary corneal epithelial cells. Cell proliferation and overall morphology were regularly monitored using a Nikon Eclipse T2000-S phase contrast microscope (Nikon Instruments Europe B. V., Amstelveen, The Netherlands). After a total of 28–33 days in differentiation culture, hPSC-LESCs were detached and seeded onto sterilized pieces of bioengineered matrix.

The sheet of bioengineered collagen matrix was cut into round pieces (1 cm in diameter) using a sterile trephine. To ensure complete removal of the storage solution, the pieces were soaked in PBS twice for 3 h, and sterilized overnight at +4 °C in a 150 U/ml penicillin/streptomycin solution (in PBS). The following day, the pieces were soaked in PBS three times for 1 h to ensure complete removal of the antibiotics. Finally, each piece was placed in a separate well of a 48-well plate, and incubated in cell culture medium overnight at +37 °C, 5% CO₂. The following day, hPSC-LESCs were treated with TrypLE Select (Invitrogen) for 5 min at +37 °C, and triturated to ensure complete detachment. Cells were pelleted by centrifugation (300 × g, 7 min), and the pellet resuspended in progenitor cell targeted CnT-20 medium (CELLnTEC Advanced Cell Systems AG). Finally, cells were seeded onto bioengineered matrices at a density of 20,000 cells/cm², and maintained in CnT-20 medium for at least 15 days. Tissue culture-treated 48-well plates coated with 5 μg/cm² human placental collagen IV were used as controls. Additionally, to test their capacity for terminal differentiation, hPSC-LESCs cultured on bioengineered matrices were further maturated in CnT-30 medium for two additional weeks.

### 2.7. Immunofluorescence

Protein expression of the pluripotency markers OCT3/4, NANOG, TRA-1-60 and TRA-1-81 in undifferentiated hPSCs, as well as protein expression of α-fetoprotein (AFP), smooth muscle actin (SMA) and Nestin in spontaneously differentiated embryoid body cultures, were analyzed using immunofluorescence, as previously described (Skottman 2010). Detection of primary antibodies (Table 1) was carried out using the following secondary antibodies, all from Molecular Probes (Thermo Fisher Scientific): donkey anti-goat IgG Alexa Fluor 488 Fluor 488 (A-11055, diluted 1:1500), goat anti-mouse IgG Alexa Fluor 568 (A-21043, diluted 1:1500) or donkey anti-mouse IgG Alexa Fluor 680 (A-10037, diluted 1:1000). Images were captured with Olympus IX51 fluorescence microscope using a 10 × objective (Olympus, Tokyo, Japan). Representative images for both cell lines are shown in Supplementary Fig. 1.

Protein expression of p63 and Ki67 was assessed before hPSC-LESC seeding and on the first day after seeding onto bioengineered matrices. To obtain a representative cell population at the time of cell seeding, a fraction of the detached cells was separated and its volume adjusted to 150 μl. Cells were then spun onto object glasses using the CellSpin I cytocentrifuge (Tharmac, GmbH, Walldoms, Germany) for quantitative analysis of protein expression. Additionally, qualitative immunofluorescence analyses were carried out to visualize protein expression of several key LESC markers (Table 1) in differentiated hPSC-LESCs. In both cases, cells were fixed and stained as previously described (Mikhailova et al., 2014). Detection of primary antibodies (Table 1) was performed using the following secondary antibodies: donkey anti-susp. IgG Alexa Fluor 488 (A-21202), donkey anti-rabbit IgG Alexa Fluor 568 (A-11042), donkey anti-rabbit IgG Alexa Fluor 488 (A-21206) or donkey anti-goat IgG Alexa Fluor 568 (A-11057), all diluted 1:800. Mounting medium containing 4',6-diamidino-2-phenylindole
Table 1
Primary antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer and catalog#</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2 [5D3]</td>
<td>Mouse</td>
<td>1:200 (5 μg/ml)</td>
<td>Millipore, MAB4155</td>
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<tr>
<td>AFP [189056]</td>
<td>Mouse</td>
<td>1:200 (2.5 μg/ml)</td>
<td>R&amp;D Systems, MAB1369</td>
</tr>
<tr>
<td>CK3 [AES]</td>
<td>Mouse</td>
<td>1:300 (2.3 μg/ml)</td>
<td>Abcam, ab77869</td>
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<tr>
<td>CK10/13 [DE-K13]</td>
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<td>1:400 (0.5 μg/ml)</td>
<td>Santa Cruz Biotech, sc-6258</td>
</tr>
<tr>
<td>CK12 [L20]</td>
<td>Goat</td>
<td>1:200 (1 μg/ml)</td>
<td>Santa Cruz Biotech, sc-17099</td>
</tr>
<tr>
<td>CK15 [LHK15]</td>
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<td>1:200 (1 μg/ml)</td>
<td>Thermo Scientific, MS-1068-P1</td>
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<td>Ki67 [polyclonal]</td>
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<td>1:500 (N/A)</td>
<td>Millipore, AB9260</td>
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<tr>
<td>Nestin [10C2]</td>
<td>Mouse</td>
<td>1:1000 (1 μg/ml)</td>
<td>Millipore, MAB5326</td>
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<tr>
<td>OCT3/4 [polyclonal]</td>
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<td>1:400 (0.5 μg/ml)</td>
<td>R&amp;D Systems, AF1759</td>
</tr>
<tr>
<td>PAX6 [polyclonal]</td>
<td>Goat</td>
<td>1:300 (N/A)</td>
<td>Sigma–Aldrich, HP125775</td>
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<tr>
<td>p40 [BC28]</td>
<td>Mouse</td>
<td>1:200 (N/A)</td>
<td>Biocare Medical, AC11066</td>
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<tr>
<td>p63 [S-16]</td>
<td>Goat</td>
<td>1:100 (2 μg/ml)</td>
<td>Santa Cruz Biotech, sc-20539</td>
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<tr>
<td>p63x2 [polyclonal]</td>
<td>Rabbit</td>
<td>1:200 (N/A)</td>
<td>Cell Signaling Tech, #4892</td>
</tr>
<tr>
<td>SMA [1A4]</td>
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<td>1:400 (1.25 μg/ml)</td>
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<tr>
<td>TCF4 [D4]</td>
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<td>TRA-1-60 [TRA-1-60]</td>
<td>Mouse</td>
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<td>Millipore, MAB4360</td>
</tr>
<tr>
<td>TRA-1-81 [TRA-1-81]</td>
<td>Mouse</td>
<td>1:400 (0.5 μg/ml)</td>
<td>Santa Cruz Biotech, sc-21706</td>
</tr>
</tbody>
</table>

(DAPI; VectaShield, Vector Laboratories Inc., Burlingame, CA) was used for visualization of nuclei. For quantitation of p63 and Ki67 expression, images of multiple randomly selected areas were captured with Olympus IX51 fluorescence microscope using a 20 × objective. Percentages of cells positive for each marker were quantified in relation to DAPI-stained cell nuclei. In the case of qualitative stainings, images were captured either with Olympus IX51 fluorescence microscope or LSM 700 confocal microscope using a 40 × objective (Carl Zeiss, Jena, Germany).

2.8. Proliferation assay

Cell viability and proliferation activity were assessed after 15 days of culture on the bioengineered matrices and control wells, using the WST-1 Cell Proliferation Assay (Takara Bio Inc., Shiga, Japan). After two PBS washes, WST-1 PreMix (diluted 1:10 in PBS) was added to each well and incubated for 4 h at +37 °C. Cell proliferation activity of the samples was evaluated by measuring the absorbance at 450 nm with a microplate reader (Victor 1429 Multilabel Counter). Duplicate samples were analyzed from each biological replicate, and empty wells were used for background normalization.

2.9. Western blotting

Human limbal epithelium was collected from cadaveric donors within 12 h post-mortem, by gently scraping the surface of the limbus. Protein expression of CK3 and CK12 in hiPSC-LESCs and native human limbal epithelium was analyzed using Western blotting, as previously described (Sorkio et al., 2014). Briefly, samples were lysed using 2 × Laemmli sample buffer, total protein separated by 7.5% SDS-PAGE and transferred onto PVDF membranes. The following primary antibodies were used: mouse anti-CK3 (1:1000, Abcam), goat anti-CK12 (1:500, Santa Cruz Biotechnology) and mouse anti-β-actin (1:2000, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-mouse and rabbit anti-goat secondary antibodies were used (both 1:3000, Santa Cruz Biotechnology), and their detection carried out with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

2.10. Statistical analyses

All values are presented as means of separate biological replicates, with error bars depicting standard deviations. Statistical analyses were carried out in the IBM SPSS Statistics Software, using the Mann–Whitney U test. Results were considered significant if p < 0.05.

2.11. Ethical issues

The use of human embryos for research purposes at Bio-MediTech, University of Tampere, has been approved by the National Authority for Medicolegal Affairs Finland (Dno 1426/32/300/05). The institute also has supportive statements of the Ethical Committee of the Pirkannaa Hospital District to derive, culture, and differentiate hESC lines (Skottman/R05116), and use hiPSC lines derived in other laboratories for ophthalmic research (Skottman/R14023). No new cell lines were derived for the purpose of this study.

Human tissue collection was carried at the University of Szeged, Hungary, with approval by the National Medical Ethics Committee (14415/2013/EKU – 183/2013 and DEOEC KEB/KEB 3094/2010), in compliance with the Helsinki Declaration guidelines (1964). Hungary follows the EU Member States’ Directive 2004/23/EC on presumed consent practice for tissue collection.

3. Results

3.1. Water content, swelling capacity and optical properties of bioengineered matrices

Water content and water uptake were studied by equilibrating the bioengineered matrices and healthy human corneas in PBS and comparing their dry and hydrated mass (Fig. 1A–B). Water content of the bioengineered matrix (91.0 ± 0.2%) was substantially higher than that of the eye bank human cornea (80.0 ± 2.5%), and a similar trend was observed for the water uptake, demonstrating that the bioengineered matrix can absorb water 9.6 times of its dry weight. This is comparable to the native human cornea, which can absorb water at about 8.5 times of its dry weight. Light transmission and scatter through bioengineered matrices at visible light wavelengths (white light, 450 nm, 550 nm, and 650 nm) was measured using custom-built optical instrument (Fig. 1C). Light transmission for 100 μm thick bioengineered matrices was over 92%, higher than the reported transmission for healthy human corneas at the same visible wavelengths (Beems and Van Best, 1990; Freigard 1997, Meek et al., 2003). Optical light scatter from bioengineered
matrices was below 4% in the visible range. Taken together, the optical properties ensure excellent transparency of the matrices—a prerequisite for a scaffold that would be transplanted onto the ocular surface.

3.2. Microstructure of bioengineered matrices

The microstructure of a freeze-dried bioengineered matrix was visualized with SEM (Fig. 2). The matrix thickness was reduced from 100 μm to about 75 μm likely due to drying and application of vacuum during imaging. The pore structure may also have been affected, although it is not possible to estimate the porosity and pore size of the matrix at this thickness. Lamellae-like structures parallel to the surface were clearly visible in the cross-section image. The bioengineered matrix had a relatively smooth surface, with salt deposits due to hydration and storage in PBS appearing as white flower-like marks.

3.3. Properties of hPSC-LESCs on bioengineered matrices

Differentiation of hPSCs was first induced towards surface ectoderm by inhibiting TGF-β and Wnt signaling pathways, and further directed towards LESC-like cells using commercial medium for corneal epithelial cell culture, CnT-30. Human PSC-LESCs expressed the LESC markers p40, p63α, ABCG2, CK15, PAX6 and TCF4, but not markers of terminally differentiated corneal epithelium CK3 and 12, or the epidermal differentiation marker CK10/13 (Fig. 3). Prior to plating onto bioengineered matrices or control wells, hPSC-LESCs possessed the appropriate polygonal morphology (Fig. 4A–B), and 52–64% of cells expressed p63 and Ki67 proteins (Fig. 4C). In case of hiPSC-LESCs, protein expression of p63 increased significantly after cell seeding: from 64% (±1%) to 78% (±7%) of p63-positive cells (p < 0.001). The same trend was observed for hESC-LESCs, but with a higher variation between replicates: 52% (±16%) and 72% (±10%) of cells were p63-positive before and after cell seeding, respectively (p < 0.05). The changes in Ki67 expression were not significant for either cell line, due to a higher inter-replicate variation. Expression of p63 and Ki67 proteins was observed clearly one day after seeding onto bioengineered matrices (Fig. 4D).

Good cell attachment and proliferation of hPSC-LESCs were observed both on control wells and on bioengineered matrices (Fig. 5A–B). After two weeks of culture, proliferation activity was significantly higher for both cell lines cultured on the bioengineered matrices, compared to the control wells (p < 0.05, Fig. 5C). Moreover, LESC markers CK15 and p63, along with the proliferation marker Ki67 were expressed at the protein level even after 30 days in culture on the bioengineered matrices in progenitor cell targeted medium CnT-20 (Fig. 5D–E). After further culture in differentiation-inducing CnT-30 medium for two weeks, hPSC-LESCs were seen to mature towards corneal epithelial cells. Proteins CK3 and CK12 were co-localized (Fig. 6A), while the expression of p63 and CK3 was mainly mutually exclusive (Fig. 6B). Protein expression of CK3 and CK12 was also analyzed using Western blotting, and both proteins were expressed at lower levels in hPSC-LESCs than in native human limbal epithelium (Fig. 6C). Finally, the construct of bioengineered matrix with mature corneal epithelial cells remained extremely transparent throughout the
course of the study (Fig. 6D).

4. Discussion

LSCD is a debilitating eye disease characterized by loss of corneal clarity and visual impairment, and its treatment remains a challenge. Patients suffering from such severe ocular surface disorders commonly undergo several surgical procedures, often failing due to corneal stromal scarring and lack of residual LESCs needed to regenerate a healthy corneal epithelium (Pellegrini et al., 2014). Restoration of a functional limbus is a key step towards successful treatment, and various strategies have been investigated with that in mind. The main challenges of cell replacement therapy using primary LESCs, or other adult stem cell sources, is their limited proliferation potential in vitro, scarcity of donor tissue, and inter-donor variability. Human PSCs may offer a solution to these issues, due to their vast differentiation potential and limitless capacity for self-renewal, providing a virtually inexhaustible supply of cells for use in tissue engineering. Several studies have demonstrated successful differentiation of hPSCs towards corneal epithelial cells (Ahmad et al., 2007; Hayashi et al., 2012; Sareen et al., 2014; Shalom-Feuerstein et al., 2012). To our knowledge, so far the only scaffolds which have been studied as carriers for hPSC-derived LESCs are decellularized porcine scaffold, human cornea, and hAM (Sareen et al., 2014; Zhu et al., 2013). However, the manufacture of such scaffolds cannot be standardized and is therefore subject to a high degree of biological variation and potential pathogen transfer.

Human AM has been demonstrated as a promising culture substrate and carrier for primary LESCs, often utilized in CLET (Baylis et al., 2011; Kolli et al., 2010). Nevertheless, hAM on its own does not sufficiently stabilize the corneal surface, permitting abnormal conjunctival invasion in the long term (Konomi et al., 2013). In addition, there are several disadvantages linked to the use of hAM, mainly its variability in thickness and biological composition, as well as poor standardization, low transparency, and suboptimal mechanical strength (Connon et al., 2010; Hopkinson et al., 2006). Aiming to replace hAM, various biomaterials have been tested as carriers for primary LESCs, most notably natural polymers such as fibrin, keratin and silk fibroin (Feng et al., 2014; Liu et al., 2012; Rama et al., 2010). The most extensively studied biomaterial for corneal applications is collagen. It is the main component of corneal stroma, has excellent biocompatibility and low immunogenicity, and has been shown to support primary LESCs and oral mucosal epithelial cells in vitro and in vivo, especially after being modified either by cross-linking or compression (Levis et al., 2010; Mi et al., 2010; Petsch et al., 2014).

In this work, we utilized a bioengineered collagen matrix that is stabilized by hybrid carbodiimide cross-linking. Similarly manufactured cell-free carriers have previously been tested in vivo as intrastromal implants, where they integrated well into the surrounding tissue, remained transparent throughout the course of the study, and proved to be biocompatible (Koulikovska et al., 2015; McLaughlin et al., 2008). The matrix has parallel lamellar microstructure and is highly cell-interactive, elastic, and fully transparent. Its high water content and swelling capacity exhibit favorable biological permeability for nutrients and cell metabolites, which is important for cell growth and cell functionality. High light transmission and low light scatter make the matrices more suitable for ocular surface reconstruction, while their elasticity and robust structure are beneficial for surgical manipulations in clinical applications.

We investigated the use of this bioengineered collagen matrix as a carrier for hPSC-LESCs. For this purpose, differentiation of hPSCs towards LESC-like cells was carried out without the use of feeder cells or serum, using a previously described method (Mikhailova et al., 2015). The obtained hPSC-LESCs possessed the appropriate epithelial cell morphology and expressed several putative LESC markers, but not CK3 or CK12, markers of terminally differentiated corneal epithelium, or CK10/13, a marker of epidermal differentiation. Before seeding onto the bioengineered matrices, protein expression of p63 was detected in an average of 52% of hESC-LESCs and 64% of hiPSC-LESCs. After seeding onto the bioengineered matrices, the amount of p63 positive cells increased to 72% and 78% of hiPSC-LESCs. After seeding onto the bioengineered matrices, the amount of p63 positive cells increased to 72% and 78% of hiPSC-LESCs and hESC-LESCs, respectively. The level of p63 expression in our study is in line with that reported in a recent study using primary LESCs cultured on compressed collagen constructs (65% ± 10%; Kureshi et al., 2014). Even after 30 days in culture on bioengineered matrices, protein expression of p63, CK15 and K67 remained high, showing that these cells retain their capacity to proliferate when in contact with the matrix, similar to transient amplifying cells in vivo. However, hPSC-LESCs were also
capable of terminal differentiation in CnT-30 medium, as evidenced by expression and co-localization of CK3 and CK12 proteins. Only about 5% of cells in the human limbus are considered to be LESCs, while the rest are likely transient amplifying cells (Pellegrini et al., 1999). Clinical studies have shown a correlation between p63 expression and LESC graft survival, with a minimum of 3% p63-positive cells greatly improving transplantation outcomes (Rama et al., 2010). Therefore, the high percentage of hPSC-LESCs remaining p63-positive in culture on the bioengineered matrices indicates an excellent potential for clinical applications. Furthermore, we have recently demonstrated using mass spectrometry-based comparative proteomics that the overall protein expression profile of hPSC-LESCs is similar to that of native ocular surface epithelium (Mikhailova et al., 2015). This evidence, together with the results of the current study, strengthens the hypothesis that hPSC-LESCs could serve as an alternative cell source for corneal reconstruction.

Currently, the mechanism through which CLET functions is not well known: the transplanted cells are either replacing the lost or damaged LESCs, or causing the patient’s own LESCs to reactivate (O’Callaghan and Daniels, 2011). Either way, it appears that stabilizing the limbal niche is crucial for successful LSCD treatment. Moreover, it is common for patients suffering from LSCD to require a corneal transplant even after the ocular surface has been stabilized through LESC transplantation, mainly due to extensive corneal stromal scarring (Kolli et al., 2010). Using a thicker carrier like the bioengineered matrix presented here may provide a solution to this problem, as it allows partial replacement of the damaged stroma in addition to restoring the epithelium. Cell-free bioengineered collagen matrices are currently under investigation in a clinical trial for the treatment of keratoconus—a disorder affecting the corneal stroma, but not the epithelium (Fagerholm et al., 2014). Although the matrices used in this study are fabricated using porcine collagen, they were shown to be non-immunogenic and well-tolerated in vivo, despite the lack of immunosuppressive medication post-transplantation (Koulikovska et al., 2015). The manufacturing technique used to produce the matrices is standardized and reproducible, minimizing biological variation. Differentiation of hPSCs towards LESC-like cells is carried out in feeder-free conditions, in a medium supplemented with serum replacement, two small molecule inhibitors and recombinant human bFGF during the surface ectoderm induction stage, followed by adherent culture on human placental collagen IV, in commercial medium developed for primary corneal epithelial cell maintenance. With minor modifications, replacing the components susceptible to biological variation with chemically-defined ones, the method can be further modified to be completely defined and xenone-free, enabling a smooth transition to clinical applications. Although xenogeneic culture components such as mouse feeder cells and calf or bovine serum might be considered safe for clinical use, they do require strict and standardized quality control (Pellegrini et al., 2014). Eliminating such problematic components from culture would simplify the process of cell differentiation and maintenance, while making it safer and more reproducible.

Finally, it is important to consider the level of differentiation of cells to be used for transplantation. A fully-stratified construct may resemble the native ocular surface more closely than a monolayer of progenitor cells, but might not be the best solution for stabilizing the damaged limbus. The results of a recent study show that tissue constructs in a more LESC-like state are better capable of re-epithelializing a wounded area than fully-stratified constructs (Massie et al., 2014). Consequently, transplantation of LESC-like cells rather than terminally differentiated corneal epithelial cells may be clinically more desirable. Here, we demonstrate that hPSC-LESCs cultured on the bioengineered collagen matrices retain their proliferative ability, while being capable of terminal differentiation, once stimulated to do so. We therefore propose that this novel tissue engineering approach may be suitable for ocular surface reconstruction, providing a sustainable corneal epithelium and partially replacing the underlying stroma. Additionally, a construct that is comprised of a bioengineered collagen matrix and mature corneal epithelial-like cells could be used as an in vitro model for drug discovery and disease modeling.

In conclusion, hPSC-LESCs are a novel source for cell-based therapy of severe ocular surface diseases. In this study, we showed that the LESC-like cells retain their capacity for proliferation and self-renewal when cultured on medical-grade bioengineered collagen matrices in serum-free conditions. Our findings suggest that hPSC-LESCs combined with the bioengineered matrices could be well-suited for clinical applications—for transplantation, or as an in vitro model of the ocular surface.

Financial disclosure

Mehrdad Rafat holds stocks and serves on the Board of Directors in a start-up/spin-off of Linköping University, LinkoCare Life Sciences AB, and is developing products related to the research being reported and holds relevant patents. The terms of his arrangements have been reviewed and approved by Linköping University in accordance with its policy on objectivity in research. No competing
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2015.11.021.

References


