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Multi-layered silk film co-culture system for human corneal epithelial and stromal stem cells

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Abstract

With insufficient options to meet the clinical demand for cornea transplants, one emerging area of emphasis is on cornea tissue engineering. In the present study, the goal was to combine the corneal stroma and epithelium into one co-culture system, in order to monitor both human corneal stromal stem cell (hCSC) and human corneal epithelial cell (hCE) growth and differentiation into keratocytes and differentiated epithelium in these 3D tissue systems *in vitro*. Co-culture conditions were first optimized, including the medium, air/liquid interface culture, and surface topography and chemistry of biomaterial scaffold films based on silk protein. The silk was used as scaffolding for both stromal and epithelial tissue layers because it is cell compatible, can be surface patterned, and is optically clear. Next, the effects of proliferating and differentiating hCEs and hCSCs in this *in vitro* system were studied, including the effects on cell proliferation, matrix formation by immunohistochemistry, and gene expression by RT-qPCR. The incorporation of both cell types into the co-culture system demonstrated more complete differentiation and growth for both cell types compared to the corneal stromal cells and corneal epithelial cells alone. Silk films for corneal epithelial culture were optimized to combine a 4.0 micron-scale surface pattern with bulk-loaded collagen type IV. Differentiation of each cell type was in evidence based on increased expression of corneal stroma and epithelial proteins and transcript levels after 6 weeks in co-culture on the optimized silk scaffolds.

Keywords

co-culture; tissue engineering; cornea; silk

1.0 Introduction

Corneal opacities are the cause about 32,400 cases of blindness and 7,640,000 cases of mild to severe visual impairment worldwide (Bourne *et al.*, 2013, Pascolini and Mariotti, 2012). Most patients undergo corneal replacement surgery to restore clarity. In the U.S., 116 000 corneas, corneal segments, and whole eyes were collected in 2014, yet only 72 000 of these

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were suitable for transplant (America, 2015). In recent years, there has been more limited availability of donor corneas due to a widening gap between donors and recipients (Gain *et al.*, 2016). As an alternative to donor cornea, synthetic keratoprosthesis are an option, however, complications can be severe (Akpek *et al.*, 2014, Ilhan-Sarac and Akpek, 2005). With no other biological options for surgery, corneal tissue engineering has become an increasingly important research focus (Ghezzi *et al.*, 2015, Lin *et al.*, 2012, Shah *et al.*, 2008).

The cornea is comprised of three distinct layers, epithelium, stroma, endothelium, and two membranes, Bowman's layer, and Descemet's membrane, respectively (DelMonte and Kim, 2011). In the present project, the focus was on the epithelium and stroma layers. The outer layer, the epithelium, protects the eye from the external environment and provides a smooth surface for the refraction of light through the cornea. The epithelium is separated from the stroma by the Bowman's layer. The stroma is about 80% of the thickness of the cornea, or 465 μm thick (Reinstein *et al.*, 2009), and ensures transparency of the cornea with a complex and mechanically robust collagen fiber organization (DelMonte and Kim, 2011). In order to engineer a biologically relevant scaffold to mimic the corneal epithelium, a biocompatible, transparent scaffold material that includes basement membrane proteins is required to allow for cell attachment and surface topography features to help with cell interactions and proliferation.

Silk fibroin is a favorable biomaterial for corneal tissue engineering due to its tunable mechanical properties (Omenetto and Kaplan, 2008, Omenetto and Kaplan, 2010), ability to maintain optical clarity long-term (Lawrence *et al.*, 2008), controllable degradation rate (Arai *et al.*, 2004, Lu *et al.*, 2011), and lack of immune response upon implantation *in vivo* (Hazra *et al.*, 2016, Meinel *et al.*, 2005, Thurber *et al.*, 2015). Silk has also been used in previous cornea tissue engineering efforts focused on the stroma using human corneal stromal stem cells (hCSCs) (Hazra *et al.*, 2016, Lawrence *et al.*, 2009, Wu *et al.*, 2014) or on the epithelium using human corneal epithelial cells (hCEs) (Bray *et al.*, 2011, Liu *et al.*, 2012). Silk solution can be cast into films (Rockwood *et al.*, 2011), and modified topographically and chemically. A recent work by Hazra et al has shown that silk fibroin films support both corneal epithelial cell and keratocyte growth, and maintain their transparency when placed *in vivo* (Hazra *et al.*, 2016). Bray et al modified the silk, studying the effect that pore size of the silk scaffolds can have on the growth of epithelial cells in a co-culture with a stromal layer (Bray *et al.*, 2012). Soft lithography or electrospinning techniques to make guided silk films affect cell attachment, proliferation, and migration (Biazar *et al.*, 2015, Gil *et al.*, 2010, Lawrence *et al.*, 2012). Furthermore, chemical modifications to silk films have enhanced cell attachment and proliferation by increasing hydrophilicity and providing cell membrane integrin binding domains for attachment (Murphy and Kaplan, 2009). These chemical modifications include surface bound RGD peptides or bulk chemical modifications, in which additional components are added to the silk solution prior to casting into films (Di Buduo *et al.*, 2015, Gil *et al.*, 2010, Murphy and Kaplan, 2009). Recent works to study silk for corneal applications have successfully incorporated chemical modifications into silk fibroin, including bulk-loaded chitosan (Guan *et al.*, 2013) and aloe vera (Kim *et al.*, 2016) and surface-coated collagen I (Kim *et al.*, 2015) to improve cell growth and function on silk fibroin. This study further builds upon previous

studies by taking advantage of the tenability of silk film scaffolds, integrating both topographical and chemical cues to develop a co-culture system focused on the stroma and epithelium.

In the present study, the goal was to design a co-culture system for human corneal epithelial and stromal stem cells in order to examine the growth and differentiation of hCSCs into keratocytes and hCEs into differentiated epithelium in 3D multilayer silk films. We hypothesized that the co-culture in this 3D system would enhance cell differentiation due to improved cell-cell signaling coupled with morphological and structural inputs. The system required the optimization of co-culture conditions and silk films for hCE growth had to be added to the previously developed stroma model (Wu *et al.*, 2014). A micron-scale pattern was added to the surface of the films, with the addition of extracellular matrix (ECM) components of the epithelial basement membrane, such as collagen type IV and fibronectin (DelMonte and Kim, 2011), to enhance hCE growth and attachment on the films. After the conditions were established, hCEs and hCSCs were combined into a 3D multilayer silk film co-culture system.

2.0 Materials and Methods

2.1 Experimental Design

To develop a viable 3D silk film system for corneal tissue engineering, the conditions for co-culture of hCSCs and hCEs were first optimized (Figure 1A). The medium composition was selected based on cell proliferation and differentiation activities. Epithelial growth factors were added to hCSC medium to allow for the culture of both cell types. Next, we investigated the differentiation of both cell types in a non-contact, co-culture model to determine if the co-culture would improve differentiation of hCSCs into keratocytes and hCEs into a differentiated epithelium (Figure 1B).

To incorporate the hCEs into the 3D silk film co-culture system, we optimized the chemistry and topography of the silk film substrates for hCE culture (Figure 1C). By adding micron-scale patterns to the surface of the films, and by bulk loading hCE-specific ECM components, hCE growth and attachment to the films was enhanced. Lastly, we studied the growth and differentiation of hCSCs into keratocytes and hCEs into a differentiated epithelium in the 3D silk film co-culture system (Figure 1D).

2.2 Cell Culture

Human corneal stromal stem cells (hCSCs) were obtained from limbal stromal tissue from corneas unfit for transplant as described previously (Hu *et al.*, 2011, Wu *et al.*, 2014). hCSCs at passage five were used for experiments. Prior to seeding, hCSCs were cultured in stem cell proliferation medium, composed of DMEM (Gibco 10569-010)/MCDB (Sigma M6770) containing 2% fetal bovine serum (Sigma F2442), 1% lipid rich bovine serum albumin (AlbuMax Gibco 11020-021), 10 ng/mL platelet derived growth factor BB (R&D Systems 520-BB), 10 ng/mL epidermal growth factor (EGF) (Sigma E9644), 0.5 mM ascorbic acid-2-phosphate (Sigma A8960), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid (ITS Gibco 41400-045), 50 µg/mL gentamicin (Gibco 15750-060), 100 µg/mL

penicillin, 100 µg/mL streptomycin (PenStrep Gibco 15140-122), 10^{-8} M dexamethasone (Sigma D4902), and 100 ng/mL cholera toxin (Sigma C8052)(Du *et al.*, 2005, Wu *et al.*, 2012). Upon reaching confluence, hCSCs were transferred to keratinocyte differentiation medium (KDM), containing Advanced DMEM (Gibco 12491-015), 0.5 mM ascorbic acid-2-phosphate, 10 ng/mL basic fibroblast growth factor (Sigma F0291), 0.1 ng/mL transforming growth factor B-3 (R&D Systems 243-BB), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin.

Human corneal epithelial cells (hCEs) were purchased from Invitrogen (Carlsbad, CA). Until seeding, hCEs were cultured in keratinocyte serum free medium (KSFM, Gibco 10724-011) with 50 µg/mL bovine pituitary extract (BPE Gibco 13028-014), 5 ng/mL EGF (Gibco 10450-013), and 1% Antibiotic/Antimycotic (Gibco 15240-062), according to manufacturer's specifications. All cells were cultured at 37°C with 5% CO₂ in humid conditions.

2.3 Determination of co-culture conditions

hCSCs and hCEs were seeded onto tissue culture plastic well plates at 50K cells/cm², using their respective growth medium (Figure 1A). The day after seeding, the medium was changed to a supplemented medium or a mixed medium, with each medium type tested on each cell type (Table 1). Supplemented medium included EGF, BPE, keratinocyte growth factor (KGF Invitrogen PHG0254), and hepatocyte growth factor (HGF Invitrogen PHG0094). These growth factors were added because of their positive influence on hCE growth (Lee *et al.*, 2001, Pancholi *et al.*, 1998, Wilson *et al.*, 1994, Wu *et al.*, 2013). The controls were hCSCs and hCEs in their respective growth medium. The cells were cultured for two weeks, with medium changes every other day. Prior to reaching confluence, the cells were cultured in stem cell growth medium that had been supplemented or mixed. Upon reaching confluence, the medium was changed to keratinocyte differentiation medium that was supplemented or mixed. Upon evaluating the initial results, select medium types were continued for six weeks to study stem cell differentiation.

Once the media were selected, the cells were cultured in a Transwell® system (Corning Inc. NY) (Figure 1B). hCSCs were seeded onto the bottom of the wells at 20K cells/cm² in stem cell proliferation medium. hCEs were seeded in KSFM onto the inserts, in separate wells, at 150K cells/cm². The difference in seeding densities accounted for the rapid proliferation of the hCSCs. On day 1, the inserts were placed into the wells with stem cells, and the medium was changed to stem cell proliferation medium with 50 µg/mL BPE, 25 ng/mL EGF, 20 ng/mL KGF, and 20 ng/mL HGF. Once the stem cells reached confluence, keratinocyte differentiation medium with 50 µg/mL BPE, 25 ng/mL EGF, 20 ng/mL KGF, and 20 ng/mL HGF was used. Once the epithelial cells reached confluence, they were exposed to an air-liquid interface culture to induce differentiation. Control wells of hCSCs alone and inserts of hCEs alone were also included.

2.4 Preparation of silk fibroin solution

The silk solution was generated as previously described (Rockwood *et al.*, 2011). Briefly, 5g of *Bombyx mori* silkworm cocoons were degummed for 30 minutes in a boiling 2L solution

of 0.02M sodium carbonate. After, the degummed fibers were dissolved in a 9.3M solution of lithium bromide for 4 hours at 60°C. The solution was then dialyzed against ultrapure water in a 3500 MW dialysis cassette for three days with regular water changes. The solution was collected from the dialysis cassettes and centrifuged twice at 12,700 g to remove any particles left in solution. The final solution collected was 6–8% (w/v).

2.5 Preparation of PDMS molds

A polydimethylsiloxane (PDMS, Dow Corning, Sylgard 184 Silicone Elastomer Kit 3097358-1004) mixture was prepared according to manufacturer's instructions. Briefly, one-part curing agent was added to nine parts of elastomer base, with rapid mixing to introduce air pockets. Patterned PDMS molds were generated by casting PDMS onto reflective diffraction gratings with different straight line groove sizes, including 0.5 μm , 2.0 μm , and 4.0 μm (Gil *et al.*, 2010, Lawrence *et al.*, 2012). Bubbles were removed in a desiccator with rapid pressure changes. The PDMS was left to cure in a 60°C oven for three hours.

2.6 Preparation of Silk Films

From this silk solution, a dilute 1% (w/v) solution was prepared. To make patterned films, polyethylene oxide (PEO $M_w = 900,000$; Sigma) was added to the solution at a concentration of 0.035%. This solution was then cast onto the PDMS molds, and dried overnight. The films were then water annealed in a 25 mmHg vacuum for 1 hour at room temperature (Yu *et al.*, 2010). The films were taped around the edges to improve ease of handling and to denote the patterned side. The films were sterilized by exposure to UV light for 30 minutes on each side. They were then soaked in Dulbecco's phosphate-buffered saline (DPBS) to leach out the PEO in films. To make the films with chemical modifications, ECM components collagen type IV (Sigma C5533) and fibronectin (Sigma F1056) were added to the silk solution at 50 $\mu\text{g/mL}$. Films that contained ECM components were water annealed for 3 hours (Hu *et al.*, 2011). The films were taped around the edges. The films were sterilized by exposure to UV light for 30 minutes on each side. Flat films with no ECM components added were used as controls.

2.7 Co-Culture System

Upon the optimization of silk film surface and bulk modifications, the samples for the 3D tissue system were prepared as follows. The dried hCE films were water annealed in a 25 mmHg vacuum for 3 hours. hCSSC films were prepared using a 1% silk, 0.035% PEO solution cast onto 4 μm groove PDMS molds. Once dry, the hCSSC films were water annealed in a 25 mmHg vacuum for 1 hour. Both film types were placed in ultrapure water for two days, with one water change, to leach out any PEO in the films. hCSSC films were then RGD treated as previously described (Wu *et al.*, 2014). Briefly, the films were equilibrated in MES buffer, pH 6.0 for 30 minutes, then placed in an EDC/NHS solution for 30 minutes. The films were then rinsed twice with MES buffer, and placed into the RGD solution for 2 hours with slight agitation. Both film types were then treated in 70% ethanol for 30 minutes for sterilization, as UV sterilization of peptide modified materials has been shown to cause degradation of peptides containing the RGD sequence (Huebsch *et al.*, 2005). The films were then rinsed 7–8 times in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco), and equilibrated in medium overnight. The sterile films were placed in 24

well plates in between two silicon tubes. The tubes had an inner diameter of 9.5 mm and an outer diameter of 15.6 mm. The films were placed in KSFM to sit for 1 hour before seeding. The films were seeded according to the co-culture conditions established in the Transwell® study, with hCEs seeded at 150K cells/cm², and hCSSCs seeded at 20K cells/cm².

After seeding, the cells were allowed to proliferate on the films. Once the cells reached 70–80% confluency, the films were stacked as described previously (Wu *et al.*, 2014). Three layers of hCSSC films were used for proof of concept. Briefly, the stem cell films were stacked with their patterns aligned orthogonally to the layer below. One hCE film was added to the top of each stack of hCSSC films. These stacks were then punched with a 12 mm biopsy and placed in 12-well plates in hCSSC proliferation medium with 25 ng/mL EGF, 20 ng/mL HGF, 20 ng/mL KGF, and 50 µg/mL BPE. A rubber ring was used to hold the films in place. When the cells reached confluence (another 2 days), the film stack was exposed to the air-liquid interface. The stack was held at the air-liquid interface using Cell Crown™ inserts for a 24-well plate. The stack was then cultured with KDM with 25 ng/mL EGF, 20 ng/mL HGF, 20 ng/mL KGF, and 50 µg/mL BPE. The medium was changed using a needle twice per week. The medium level was checked daily to ensure that the top film of the stack was at the air-liquid interface. The samples were cultured for three and six weeks at 37°C, 5% CO₂ in humid conditions.

2.8 Cell Proliferation Assessment

Cell proliferation activity was quantified using alamarBlue® (Invitrogen DAL1100) in both of the medium co-culture condition determinations. For each test, 4 replicates per sample were used. Briefly, 10% alamarBlue® solution in the culture medium was added to each well incubated at 37°C for 4 hours. Control wells without cells were used as blanks. Three medium samples were then collected per well and fluorescent measurements were taken with 550 nm excitation and 590 nm emission. The samples for each well were averaged and then normalized to day 1 measurements. Assays were completed on days 1, 3, 6, 10, 14 for medium determination and initial Transwell co-culture. In the case of the co-culture wells, the epithelial inserts were incubated separately from the stem cell wells.

2.9 Cell Adhesion Assay

Cell attachment to the films was assessed using a cell adhesion assay. hCEs were seeded onto the films prepared according to the method in 2.7. Five replicates per film type per time point were imaged (15 per type overall). Films were imaged at 45 minutes, 1.5 hours, and 3 hours after seeding. At each time point, the medium was removed to collect any cells that did not attach to the films. Films were then rinsed once with DPBS. Between 9 and 12 phase contrast images of each film were taken using a Keyence BZ-X700 microscope (Keyence, Itasca, IL) to account for the entire 8 mm diameter film, and stitched together using the Keyence BZ-X Analyzer software. Cell selection and area were determined automatically using the Keyence BZ-X Analyzer software. ANOVA was performed to evaluate differences in cell coverage on the films depending on film types. Statistical significance was given for $p < 0.05$.

2.10 Cell Phenotype Assessment

Cell differentiation was evaluated using immunocytochemistry. The control hCSSC and hCE wells were fixed using 2.5% paraformaldehyde for 20 minutes. The films were fixed for 30 minutes, and stacked films were fixed for 1 hour. After rinsing with PBS, the films were blocked with horse serum to prevent non-specific binding. hCSSCs were then incubated with anti-BCRP (Abcam ab3380) at 1:100 primary antibodies for the two week time point to visualize loss of stem cell phenotype (Gil *et al.*, 2010). The films were also analyzed with anti-keratocan (Santa Cruz sc-33243) at 1:50, anti-keratan sulfate (Santa Cruz sc-73518) at 1:50, anti-decorin (Abcam ab54728) at 1:50, and anti-lumican (Abcam ab168348) at 1:50 primary antibodies for the six week time point to visualize hCSSC differentiation into keratocytes (Lawrence *et al.*, 2009). hCEs were incubated with anti-involucrin (Abcam ab80530) at 1:500 and anti-connexin 43 (Abcam ab11370) at 1:1 000 primary antibodies to show the change to a differentiated epithelium (Yu *et al.*, 2010). After rinsing, the wells were incubated with fluorescently tagged secondary antibodies, including TRITC anti-rabbit (Abcam ab96894), FITC anti-mouse (Abcam ab7064), and FITC anti-goat (Abcam ab6881) at 1:500, and DAPI at 1:10, and then imaged using the Keyence fluorescence microscope and the z-stack function, which allows for imaging up to 150 μm . Images were taken of the entire thickness and stacked together.

2.11 Reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

hCSSC and hCE expression levels of various genes were assessed by real-time quantitative Reverse Transcription–Polymerase Chain Reaction (RT-qPCR). At 6 weeks, total RNA was extracted from the hCSSCs and hCEs using a RNeasy mini kit (Qiagen, Valencia, CA) and homogenized using QIAshredders (Qiagen, Valencia, CA) according to the manufacturer's instructions. After RNA separation, the concentration and integrity of the collected nucleic acid were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA). Reverse transcription of total RNA (250 ng) was used to generate complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit ABI Biosystems (Life Technologies, Grand Island, NY) according to manufacturer's instructions. RT-qPCR was completed using a MX3000P (Stratagene, Agilent Technologies, Santa Clara, CA). Each PCR reaction was completed with 4 μL of cDNA, 5 μL of RNase-free water, 1 μL of TaqMan® Gene Expression Assay, and 10 μL of TaqMan® Gene Expression Master Mix (Life Technologies, Grand Island, NY). The TaqMan® probes were lumican (Hs00929860_m1), PDK4 (Hs00176875_m1), PTDGS (Hs00168748_m1), ALDH3A1 (Hs00964880_m1), α SMA (smooth muscle actin) (Hs00426835_g1), and reference gene ribosomal RNA 18S (Hs99999901_s1) for hCSSCs, while KRT3 (keratin 3, Hs00365074_m1), IVL (involucrin, Hs00846307_s1), GJA4 (gap junction protein alpha 4, Hs00704917_s1), and reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Hs02758991_g1) were used for hCEs. PCR conditions were cycled: 50°C for 2 min, the initial denaturation at 95°C for 10 min, and then 50 cycles of 15 seconds at 95°C then 1 min at 60°C. Relative quantification of the target gene expression was achieved by first normalizing against an endogenous reference gene (18S for hCSSCs, and GAPDH for hCEs) to correct for differences in input RNA, and then relating the expression of the target genes at 6 weeks to the expression at day 1 using the $-2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

3.0 Results

Co-Culture Condition Optimization

3.1 Cell Proliferation—Cell proliferation of hCEs and hCSSCs were measured separately using alamarBlue® fluorescence (Figure 2). Higher proliferative activity was shown for hCSSCs cultured in 100% proliferation medium (Table 1) than in the other supplemented media (Figure 2B). Higher proliferative activity was also shown for hCEs in 100% proliferation medium compared to the other supplemented media (Figure 1B). hCE proliferation activity was higher than hCSSC proliferation regardless of medium type.

Co-Culture Using Transwell Plates

3.2 Cell Proliferation Assessment—Cell proliferation of hCEs and hCSSCs was measured in a Transwell® system using alamarBlue® fluorescence (Figure 3). When proliferation medium alone was used (Figure 3A), the proliferation activity of the hCSSCs in co-culture with hCEs was lower than hCSSCs cultured independently at each day activity was measured. When the proliferation medium was switched to differentiation medium after the hCSSCs reached confluence on day 5 (Figure 3B), there was a decrease in proliferation activity for both cell types.

3.3 Cell Phenotype Assessment—Change in phenotype of hCEs and hCSSCs from co-culture conditions was observed using immunohistochemistry (Figures 4, S1, and S2). hCEs were stained with differentiated epithelial markers connexin 43, involucrin, and cell nuclei marker DAPI. On day 1 of culture, only cell nuclei staining was visible (Figure 4, Figure S1). After 14 days in culture, both the hCEs cultured independently and hCEs in co-culture stained positive for both differentiated epithelial markers. Compared visually to the hCE only culture, more connexin 43 was present in the hCEs co-cultured with hCSSCs. hCSSCs were stained with stem cell marker BCRP and cell nuclei marker DAPI. On day 1, BCRP was visible in the cells (Figure 4, Figure S2). After 14 days in culture, both the hCSSCs cultured independently and hCSSCs in co-culture visually showed decreased BCRP signal. Compared visually to the hCSSCs culture only, the hCSSCs co-cultured showed lower content of the stem cell marker.

Silk Film Optimization for hCEs

3.4 hCE Adhesion—A cell attachment study showed that hCE adhesion had both a time and a sample dependent effect. Analysis of variance (ANOVA) was completed on the data against the flat films and the plain silk films to assess the difference in cell coverage between the measurements taken at each time point. The cell area coverage on each film type was different depending on the pattern as well as the bulk-loaded ECM components. In the case of the 4.0 µm films, after allowing the cells to incubate 3 hours on the films, there was a significantly higher cell coverage of the film compared to the other patterns tested ($p < 0.05^*$) (Figure 5A). The cell area coverage on ECM-loaded films was significantly different for collagen type IV and collagen type IV + fibronectin compared to the control and fibronectin films ($p < 0.05^*$) after the 3 hour incubation (Figure 5B).

Silk Film Co-Culture System Study

3.5 Long-term Cell Phenotype—Cell phenotype of the 3D samples was analyzed after 6 weeks in culture using PCR and immunocytochemistry (Figures 6 and 7). After 6 weeks, hCEs had high levels of protein expression for hCE differentiation makers involucrin and connexin 43 as shown in z-stacks of the entire construct in Figures 6A and S3, and higher levels of transcript for hCE differentiation markers keratin 3 relative to day 1 (Figure 6B). High levels of protein expression for keratocyte markers decorin, lumican, keratin sulfate, and keratocan were observed in constructs after 6 weeks in the hCSSC cultures (Figure 7A, S4). Higher transcript expression of keratocyte marker ALDH3A1 was seen compared to expression at day 1 (Figure 7B).

4.0 Discussion

Due to the widening gap between donors available and patients on the waiting list for transplants, tissue engineered cornea has become an important research target. When designing a tissue-engineered cornea, both the biomaterials and the cells used must be carefully considered to ensure a suitable design. For this study, silk, a transparent protein with tunable mechanical and degradation properties (Arai *et al.*, 2004, Lawrence *et al.*, 2008, Omenetto and Kaplan, 2008, Omenetto and Kaplan, 2010), was cast into patterned films to direct hCSSC and hCE differentiation into keratocytes and differentiated epithelium. Building upon our previous study where we generated bioengineered corneal stromal tissue (Wu *et al.*, 2014), we sought to expand the cell types of system complexity in the present work. The stroma, as the most complicated portion of the cornea, with highly organized collagen ECM comprised of lamellae to maintain optical clarity (DelMonte and Kim, 2011), was studied first in our prior effort. The engineered stroma was designed to mimic the mechanical strength and the optical clarity found *in vivo* (Wu *et al.*, 2014). We hypothesized that by adding the corneal epithelial layer, interactions between these cell types would allow for more complete differentiation of hCSSCs and hCEs, which would better mimic the native cornea.

The first objective for the co-culture system was to develop a medium that would allow for the culture of both cell types. We focused on maintaining the proliferation and differentiation functions of each cell type. After adding epithelial growth factors, including EGF, HGF, KGF, and BPE (Lee *et al.*, 2001, Pancholi *et al.*, 1998, Wilson *et al.*, 1994, Wu *et al.*, 2013, Yu *et al.*, 2010), to the stem cell medium, high proliferative activity was observed for both hCEs and hCSSCs in stem cell proliferation medium. From these results, we selected the 100% growth factor medium with 25 ng/mL EGF, 20 ng/mL HGF, 20 ng/mL KGF, and 50 µg/mL BPE added.

After the co-culture medium was established, the remaining conditions were determined by co-culturing hCEs and hCSSCs in Transwell® plates. We optimized seeding densities to match the growth of each cell type and moved hCEs to the air-liquid interface once they formed a monolayer. Using this system, the changes in growth and differentiation of hCSSCs and hCEs as a result of the co-culture were evaluated.

Changes in cell proliferation as a result of the co-culture conditions were evident from alamarBlue® studies. Two different culture conditions were studied; co-culture proliferation medium only and co-culture proliferation medium switched to co-culture differentiation medium on day 5. With only the co-culture proliferation medium, the proliferation activity of the hCSSC in co-culture was reduced compared to the hCSSCs cultured independently, suggesting that the hCEs stimulated the hCSSCs to switch from proliferation to differentiation. After switching to co-culture differentiation medium, a difference in hCE proliferation activity was also evident, as the co-culture also showed lower proliferative activity than those cultured independently. This suggests that the hCEs were stimulated by the hCSSCs to begin differentiation activity earlier.

To show changes in differentiation due to co-culture, differentiation epithelial markers, stem cell markers, and keratocyte markers were examined. There was no nonspecific staining observed at the initial time points. Staining for both the hCEs alone and the hCEs in co-culture showed formation of tight junctions over the two-week culture period. While both the hCEs alone and the hCEs in co-culture expressed these markers, the hCEs in co-culture had higher expression of these markers, suggesting that these hCEs were able to form multilayers more quickly. This would be expected, as corneal epithelial cells form a stratified squamous epithelium *in vivo* (DelMonte and Kim, 2011). More of the junction markers were present in the co-cultured hCE samples, which suggested that signaling between the hCSSCs and the hCEs helped the hCEs to form a thicker multilayer more quickly than when the hCEs were cultured alone. Stem cell marker BCRP, present in proliferating cells, was used to stain hCSSC samples. While this marker was clearly visible on day 1, nearly all expression was lost after two weeks in culture. Compared to the hCSSCs cultured independently, those in co-culture showed less of the stem cell marker after 14 days, indicating that the hCEs communication promoted a faster transition to differentiation activities.

After the co-culture conditions were characterized, the 3D co-culture system was developed. First, the silk film scaffolds for hCE cultures were optimized by using bulk ECM loading and surface topography changes to promote cell adhesion and growth. We hypothesized that by adding proteins common in the basement membrane of the corneal epithelium, including collagen type IV, fibronectin, and the combination (DelMonte and Kim, 2011), we would be able to improve hCE adhesion, proliferation, and differentiation. After completing statistical analysis on cell adhesion data, collagen type IV was the most effective at enhancing hCE cell attachment.

The film patterns tested were based on work previously done in this laboratory that indicated that patterns on the same order of magnitude as the cell size affect cell spreading and alignment (Gil *et al.*, 2010, Lawrence *et al.*, 2012). This work showed that cells in circular patterns tended to be 15% smaller near the center of the circle when compared to straight-lined samples (Lawrence *et al.*, 2012). For this reason, we chose to test linear-patterned samples only. Three different pattern sizes were tested for enhanced hCE adhesion, proliferation, and differentiation, and were then compared to flat film surfaces. Given previous results obtained with patterned films, we expected that at least one of the patterns would show elevated cell growth compared to the flat films (Gil *et al.*, 2010, Lawrence *et al.*,

2012). After analyzing samples at each time point to examine the cell adhesion, the 4.0 μm films were the only films with significantly more cells than the flat films at the 3-hour time point. Once the silk film surface and chemistry were optimized for hCE cultures, the hCE films were combined with the hCSSC films developed previously in this lab (Wu *et al.*, 2014) to generate a more biomimetic engineered cornea-like tissue. To evaluate the effectiveness of this 3D co-culture system, we tested the co-cultured film samples using PCR and immunochemistry. In the case of both the hCSSCs and the hCEs, increased expression of differentiation markers was observed over 6 weeks in culture. The differentiation of hCEs was visible through protein expression of involucrin and connexin 43, and an increase in mRNA expression of keratin 3. The hCSSCs showed differentiation to keratocytes through protein expression of keratocan, keratin sulfate, lumican, and decorin, and an increase in mRNA expression of ALDH3A1. These results suggested that the co-culture of these cell types in this system allowed for differentiation of hCEs and hCSSCs to the appropriate cell types for each layer of the cornea.

While we developed a partial thickness cornea tissue model that included stroma and epithelium, there are some remaining limitations. For example, this cornea tissue model does not include an endothelial layer, which is important in cases where the entire thickness of the cornea is necessary, as in the case of transplants. Furthermore, when testing future versions of the system, comparisons between this model and other cornea models will be useful to highlight utility in comparison to native cornea tissue.

However, while this co-culture system only represents a partial thickness cornea by incorporating the stroma and the epithelium, it has the potential to be used in several applications. First, in cases where corneal damage does not penetrate the entire cornea, it could be used as a partial thickness replacement. In addition, by incorporating the cornea's epithelial and stromal layers, it could be used for modeling the cornea to study other diseases experienced at the cornea. For instance, this co-culture system could be used to study bacterial or fungal movement through the epithelium into the stroma, as in the case of infectious keratitis, which can cause damage to both layers of the cornea (Bourcier *et al.*, 2003, Green *et al.*, 2008). By damaging the cornea, infectious keratitis can cause vision loss, and can require corneal transplants to correct (Gain *et al.*, 2016). The next step for this corneal replacement would be to incorporate the endothelial layer, which would allow a full thickness corneal tissue replacement. A full thickness cornea would be helpful in studying additional corneal infections *in vitro*, but would more importantly address the world wide need for corneal replacements.

5.0 Conclusions

A co-culture system for 3D tissue engineering of the cornea, including both the corneal stroma and the corneal epithelium, was developed. Using corneal ECM bulk-loaded, patterned silk films, hCEs that could be added to a tissue engineered corneal stroma developed previously in this lab using silk films and hCSSCs, was co-cultured. This process also included the optimization of the co-culture medium and co-culture conditions to ensure growth and differentiation of both hCEs to a differentiated epithelium and hCSSCs to keratocytes. This 3D co-culture system was the next step towards the generation of a full-

thickness corneal tissue replacement. With the addition of the endothelial layer to this 3D tissue engineered cornea as a next step, a more complete cornea would result.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Akpek EK, Alkharashi M, Hwang FS, et al. Artificial corneas versus donor corneas for repeat corneal transplants. Cochrane Database of Systematic Reviews. 2014
- America EBAO. 2014 Eye Banking Statistical Report. 2015. from http://www.restore sight.org/wp-content/uploads/2015/03/2014_Statistical_Report-FINAL.pdf
- Arai T, Freddi G, Innocenti R, et al. Biodegradation of Bombyx mori silk fibroin fibers and films. Journal of Applied Polymer Science. 2004; 91:2383–2390.
- Biazar E, Baradaran-Rafii A, Heidari-keshel S, et al. Oriented nanofibrous silk as a natural scaffold for ocular epithelial regeneration. Journal of Biomaterials Science-Polymer Edition. 2015; 26:1139–1151. [PubMed: 26324020]
- Bourcier T, Thomas F, Borderie V, et al. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. British Journal of Ophthalmology. 2003; 87:834–838. [PubMed: 12812878]
- Bourne RRA, Stevens GA, White RA, et al. Causes of vision loss worldwide, 1990–2010: a systematic analysis. Lancet Global Health. 2013; 1:E339–E349. [PubMed: 25104599]
- Bray LJ, George KA, Ainscough SL, et al. Human corneal epithelial equivalents constructed on Bombyx mori silk fibroin membranes. Biomaterials. 2011; 32:5086–5091. [PubMed: 21501866]
- Bray LJ, George KA, Hutmacher DW, et al. A dual-layer silk fibroin scaffold for reconstructing the human corneal limbus. Biomaterials. 2012; 33:3529–3538. [PubMed: 22336295]
- DelMonte DW, Kim T. Anatomy and physiology of the cornea. Journal of Cataract & Refractive Surgery. 2011; 37:588–598. [PubMed: 21333881]
- Di Buduo CA, Wray LS, Tozzi L, et al. Programmable 3D silk bone marrow niche for platelet generation ex vivo and modeling of megakaryopoiesis pathologies. Blood. 2015; 125:2254–2264. [PubMed: 25575540]
- Du Y, Funderburgh ML, Mann MM, et al. Multipotent Stem Cells in Human Corneal Stroma. Stem cells (Dayton, Ohio). 2005; 23:1266–1275.
- Gain P, Jullienne R, He ZG, et al. Global Survey of Corneal Transplantation and Eye Banking. Jama Ophthalmology. 2016; 134:167–173. [PubMed: 26633035]
- Ghezzi CE, Rnjak-Kovacina J, Kaplan DL. Corneal Tissue Engineering: Recent Advances and Future Perspectives. Tissue Engineering Part B: Reviews. 2015; 21:278–287. [PubMed: 25434371]
- Gil ES, Mandal BB, Park S-H, et al. Helicoidal multi-lamellar features of RGD-functionalized silk biomaterials for corneal tissue engineering. Biomaterials. 2010; 31:8953–8963. [PubMed: 20801503]
- Gil ES, Park S-H, Marchant J, et al. Response of Human Corneal Fibroblasts on Silk Film Surface Patterns. Macromolecular Bioscience. 2010; 10:664–673. [PubMed: 20301120]
- Green M, Apel A, Stapleton F. Risk factors and causative organisms in microbial keratitis. Cornea. 2008; 27:22–27. [PubMed: 18245962]
- Guan L, Ge HY, Tang XL, et al. Use of a Silk Fibroin-Chitosan Scaffold to Construct a Tissue-Engineered Corneal Stroma. Cells Tissues Organs. 2013; 198:190–197. [PubMed: 24247045]
- Hazra S, Nandi S, Naskar D, et al. Non-mulberry Silk Fibroin Biomaterial for Corneal Regeneration. Scientific Reports. 2016; 6:13. [PubMed: 28442757]

- Hu X, Shmelev K, Sun L, et al. Regulation of Silk Material Structure by Temperature-Controlled Water Vapor Annealing. *Biomacromolecules*. 2011; 12:1686–1696. [PubMed: 21425769]
- Huebsch N, Gilbert M, Healy KE. Analysis of sterilization protocols for peptide-modified hydrogels. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*. 2005; 74B:440–447.
- Ilhan-Sarac O, Akpek EK. Current concepts and techniques in keratoprosthesis. *Current Opinion in Ophthalmology*. 2005; 16
- Kim DK, Sim BR, Khang G. Nature-Derived Aloe Vera Gel Blended Silk Fibroin Film Scaffolds for Cornea Endothelial Cell Regeneration and Transplantation. *ACS Applied Materials & Interfaces*. 2016; 8:15160–15168. [PubMed: 27243449]
- Kim EY, Tripathy N, Cho SA, et al. Bioengineered neo-corneal endothelium using collagen type-I coated silk fibroin film. *Colloids and Surfaces B-Biointerfaces*. 2015; 136:394–401.
- Lawrence BD, Cronin-Golomb M, Georgakoudi I, et al. Bioactive Silk Protein Biomaterial Systems for Optical Devices. *Biomacromolecules*. 2008; 9:1214–1220. [PubMed: 18370418]
- Lawrence BD, Marchant JK, Pindrus M, et al. Silk film biomaterials for cornea tissue engineering. *Biomaterials*. 2009; 30:1299–1308. [PubMed: 19059642]
- Lawrence BD, Pan Z, Liu A, et al. Human Corneal Limbal-Epithelial Cell Response to Varying Silk Film Geometric Topography In Vitro. *Acta biomaterialia*. 2012; 8:3732–3743. [PubMed: 22705042]
- Lee JS, Liu JJ, Hong JW, et al. Differential expression analysis by gene array of cell cycle modulators in human corneal epithelial cells stimulated with epidermal growth factor (EGF), hepatocyte growth factor (HGF), or keratinocyte growth factor (KGF). *Current Eye Research*. 2001; 23:69–76. [PubMed: 11821989]
- Lin J, Yoon K-C, Zhang L, et al. A Native-Like Corneal Construct Using Donor Corneal Stroma for Tissue Engineering. *PLoS ONE*. 2012; 7:e49571. [PubMed: 23166715]
- Liu J, Lawrence BD, Liu A, et al. Silk Fibroin as a Biomaterial Substrate for Corneal Epithelial Cell Sheet GenerationSF as a Biomaterial Substrate. *Investigative Ophthalmology & Visual Science*. 2012; 53:4130–4138. [PubMed: 22661480]
- Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2⁻CT Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Lu QA, Zhang B, Li MZ, et al. Degradation Mechanism and Control of Silk Fibroin. *Biomacromolecules*. 2011; 12:1080–1086. [PubMed: 21361368]
- Meinel L, Hofmann S, Karageorgiou V, et al. The inflammatory responses to silk films in vitro and in vivo. *Biomaterials*. 2005; 26:147–155. [PubMed: 15207461]
- Murphy AR, Kaplan DL. Biomedical applications of chemically-modified silk fibroin. *Journal of materials chemistry*. 2009; 19:6443–6450. [PubMed: 20161439]
- Omenetto FG, Kaplan DL. A new route for silk. *Nat Photon*. 2008; 2:641–643.
- Omenetto FG, Kaplan DL. New Opportunities for an Ancient Material. *Science (New York, N.Y.)*. 2010; 329:528–531.
- Pancholi S, Tullo A, Khaliq A, et al. The effects of growth factors and conditioned media on the proliferation of human corneal epithelial cells and keratocytes. *Graefes Archive for Clinical and Experimental Ophthalmology*. 1998; 236:1–8.
- Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. *The British Journal of Ophthalmology*. 2012; 96:614–618.
- Reinstein DZ, Archer TJ, Gobbe M, et al. Stromal Thickness in the Normal Cornea: Three-dimensional Display With Artemis Very High-frequency Digital Ultrasound. *Journal of Refractive Surgery*. 2009; 25:776–786. [PubMed: 19772263]
- Rockwood DN, Preda RC, Yucel T, et al. Materials fabrication from Bombyx mori silk fibroin. *Nat. Protocols*. 2011; 6:1612–1631. [PubMed: 21959241]
- Shah A, Brugnano J, Sun S, et al. The Development of a Tissue-Engineered Cornea: Biomaterials and Culture Methods. *Pediatr Res*. 2008; 63:535–544. [PubMed: 18427299]
- Thurber AE, Omenetto FG, Kaplan DL. In vivo bioresponses to silk proteins. *Biomaterials*. 2015; 71:145–157. [PubMed: 26322725]

- Wilson SE, He Y-G, Weng J, et al. Effect of Epidermal Growth Factor, Hepatocyte Growth Factor, and Keratinocyte Growth Factor, on Proliferation, Motility and Differentiation of Human Corneal Epithelial Cells. *Experimental Eye Research*. 1994; 59:665–678. [PubMed: 7698260]
- Wu J, Du Y, Mann MM, et al. Bioengineering Organized, Multilamellar Human Corneal Stromal Tissue by Growth Factor Supplementation on Highly Aligned Synthetic Substrates. *Tissue Engineering. Part A*. 2013; 19:2063–2075. [PubMed: 23557404]
- Wu J, Du Y, Watkins SC, et al. The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells. *Biomaterials*. 2012; 33:1343–1352. [PubMed: 22078813]
- Wu J, Rnjak-Kovacina J, Du Y, et al. Corneal stromal bioequivalents secreted on patterned silk substrates. *Biomaterials*. 2014; 35:3744–3755. [PubMed: 24503156]
- Yu F-SX, Yin J, Xu K, et al. Growth factors and corneal epithelial wound healing. *Brain research bulletin*. 2010; 81:229–235. [PubMed: 19733636]

A. Medium Optimization **B. Transwell Co-Culture** **C. hCE Film Optimization** **D. Silk Scaffold Co-Culture** **Figure 1. Schematic of the tissue engineered cornea experimental design**

(A) Medium was optimized for growth of both hCEs and hCSCCs by testing combinations of hCSCC medium and hCE medium, as well as hCSCC medium with hCE growth factors (GF) added. (B) hCEs and hCSCCs were cultured in Transwell® systems to study the effects of the co-culture on the proliferation and differentiation of each cell type. (C) Silk film topography and chemistry were optimized for hCE growth. Different micron-scale groove sizes and extracellular matrix proteins were tested to enhance cell attachment and growth.

(D) Tissue engineered corneas containing hCE films and hCSSC films were cultured long term in optimized medium.

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Figure 2. AlamarBlue® results for co-culture medium optimization

Proliferation activity was measured using fluorescence intensity and normalized to day 1 and compared using two-way ANOVA. (A) hCEs showed significantly higher proliferation activity in all media compared to human keratinocyte serum free medium (Ep). Growth factor added media (40, 70, 100%) were compared to stem cell proliferation medium (Stem). hCE proliferation activity in 100% supplemented medium showed no significant differences to stem cell proliferation medium, but lower proliferation activity was seen at Day 9 for the 70% and 40% supplemented media ($p < 0.05^*$, $p < 0.0001^{****}$, respectively). When comparing to the 100% supplemented medium, hCE proliferation activity was lower at Day 9 for the 40% supplemented medium ($p < 0.01^{++}$). (B) hCSSCs showed higher proliferation activity at Day 9 with the hCSSCs cultured in the 100% supplemented medium when compared to stem cell proliferation medium ($p < 0.0001^{****}$). When compared to the cells cultured in 100% supplemented medium, hCSSCs cultured in 70% and 40% supplemented media showed lower proliferation activity ($p < 0.001^{+++}$ and $p < 0.0001$, respectively). Normal stem cell proliferation medium and human keratinocyte serum free medium were used as control media. Error bars are shown as $\pm$ standard deviation.

Figure 3. alamarBlue® results for the Transwell® co-culture system

Proliferation activity was measured using fluorescence intensity and normalized to day 1 and compared using two-way ANOVA. (A) hCSSCs in co-culture showed decreased proliferation compared to hCSSCs cultured independently ($p < 0.01^{**}$, $p < 0.0001^{****}$), without the switch to differentiation medium plus growth factors. (B) After the medium was switched to differentiation medium plus growth factors (day 5), there was a decrease in proliferative activity of both hCEs and hCSSCs in co-culture.

Figure 4.

(A) Immunohistochemical staining of hCEs in culture using connexin 43 (red), involucrin (green), and DAPI (blue). At day 14, hCEs in co-culture expressed more of the marker present in differentiated epithelium than hCEs cultured independently. (B) Immunohistochemical staining of hCSSCs in culture using BCRP (green), and DAPI (blue). At day 14, hCSSCs in culture expressed less of the stem cell marker.

Figure 5. Cell Adhesion assay results for surface topography and ECM addition, showing statistical differences between time points and samples

Cell adhesion results were analyzed using two-way ANOVA. (A) 3 hours after seeding with hCEs, the films with 4.0 μm grooves retained the most cells compared to the other groove types ($p < 0.05$). (B) 3 hours after seeding with hCEs, the films prepared with collagen type IV retained the most cells compared to the other topography chemistries ($p < 0.05$).

Figure 6. hCE differentiation in 3D culture

(A) hCEs cultured in 3D with hCSCs for 6 weeks showed high protein expression of involucrin and connexin 43 through immunostaining. (B) RT-qPCR results were analyzed relative to a housekeeping gene and normalized to day 1. One-way ANOVA was used to compare transcript expression to day 1 values. hCEs cultured in 3D with hCSCs for 6 weeks were evaluated for keratin 3 (KRT3), $n=4$, involucrin (IVL), $n=3$, and gap junction protein alpha 4 (GJA4), $n=4$, and showed a significant increase in mRNA expression of KRT3 over the course of 6 weeks ($p<0.0001$ ****).

Figure 7. hCSSC differentiation in 3D culture

(A) hCSSCs cultured in 3D with hCEs for 6 weeks showed high protein expression of keratin sulfate, keratocan, decorin, and lumican. (B) RT-qPCR results were analyzed relative to a housekeeping gene and normalized to day 1. One-way ANOVA was used to compare transcript expression to day 1 values. hCSSCs cultured in 3D with hCEs for 6 weeks were evaluated for stroma markers lumican (Lum), pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4), lipocalin prostaglandin D synthase (PTDGS), aldehyde dehydrogenase 3 family member A1 (ALDH3A1), and alpha smooth muscle actin (aSMA), and showed a

significant increase in mRNA expression of ALDH3A1 over the course of 6 weeks ($p < 0.001^{***}$).

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Table 1

Types of Medium Tested for Co-Culture

SUPPLEMENTED MEDIA		MIXED MEDIA	
Stem Cell medium supplemented with BPE, EGF, KGF, and HGF		Stem Cell medium mixed with KSFM (based on volume)	
40%	20 µg/mL BPE, 10 ng/mL EGF, 8 ng/mL KGF, 8 ng/mL HGF	25%	hCSSC medium, 75% KSFM
70%	35 µg/mL BPE, 17.5 ng/mL EGF, 14 ng/mL KGF, 14 ng/mL HGF	50%	hCSSC medium, 50% KSFM
100%	50 µg/mL BPE, 25 ng/mL EGF, 20 ng/mL KGF, 20 ng/mL HGF	75%	hCSSC medium, 25% KSFM

Table 2

Films tested for epithelial cell culture

Patterns Tested	ECM Added	PEO Added
0.5 um grooves	No	Yes
2.0 um grooves	No	Yes
4.0 um grooves	No	Yes
No	No	Yes
No	Collagen IV	No
No	Fibronectin	No
No	Collagen IV + Fibronectin	No
No	No	No