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An in vitro 3-dimensional Collagen-based Corneal Construct with Innervation Using Human Corneal Cell Lines

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Purpose: To develop a 3-dimensional corneal construct suitable for in vitro studies of disease conditions and therapies.

Design: In vitro human corneal constructs were created using chemically crosslinked collagen and chondroitin sulfate extracellular matrix and seeded with 3 human corneal cell types (epithelial, stromal, and endothelial) together with neural cells. The neural cells were derived from hybrid neuroblastoma cells and the other cells used from immortalized human corneal cell lines. To check the feasibility and characterize the constructs, cytotoxicity, cell proliferation, histology, and protein expression studies were performed.

Results: Optimized culture condition permitted synchronized viability across the cell types within the construct. The construct showed a typical appearance for different cellular layers, including healthy appearing, phenotypically differentiated neurons. The expected protein expression profiles for specific cell types within the construct were confirmed with western blotting.

Conclusions: An in vitro corneal construct was successfully developed with maintenance of individual cell phenotypes with anatomically correct cellular loci. The construct may be useful in evaluation of specific corneal disorders and in developing different corneal disease models. Additionally, the construct can be used in evaluating drug targeting and/or penetration to individual corneal layers, testing novel therapeutics for corneal diseases, and potentially reducing the necessity for animals in corneal research at the early stages of investigation.

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Currently, basic understanding of corneal biology and research addressing corneal diseases are largely dependent on in vivo animal studies. However, in numerous cases, the dissimilarities in anatomy and physiology between human and nonhuman animal eyes have led to outcomes that do not effectively translate into human clinical therapeutic trials.[1,2] An alternative and additive approach to experimental modeling of a disease state by creating organ facsimiles is emerging. In this approach, a particular organ is mimicked in vitro with a 3-dimensional (3D) model with appropriate microenvironment, typically containing cells of human origin. Such approaches, which include spheroids, organoids[3] and organ-on-a-chip,[4] can be highly tractable in that they permit wide spectrum molecular intervention and experimental manipulation of internal and external conditions that would otherwise be practically impossible in an in vivo disease model. For the cornea, there have been a few notable successes in which a 3D corneal model was used to better understand normal corneal physiology and/or to mimic a disease condition.[5–8] Cell-based models have been developed for the study of drug transport,[9] drug metabolism[10,11] and toxicity.[12] Collagen matrix-based 3D corneal models[5,13–16] have been developed to examine corneal nerve-target cell interactions[7] and drug permeability.[16–18] However, the unique and distinct function of individual cell types and their required dependencies for specific local and regional environments remain an unmet goal.

The cornea is unique in that it is highly innervated with 300- to 600-fold more sensory neurons than the skin[19] and is also avascular. It contains 3 major layers: the outermost stratified epithelium, the middle stromal layer populated mostly by keratocytes, and an innermost single layer of endothelial cells.[20] The distinct corneal cell types play critical roles in its function, physiology, and, consequently, corneal health and disease. The corneal epithelium provides a physical barrier to injury and infection and plays a key role in corneal clarity.[21]

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Keratocytes maintain the corneal extracellular matrix[1] and control its transparency.[22] Crosstalk between keratocytes and corneal epithelial cells during injury and wound healing determines the degree of secondary fibrosis and opacity.[23] Corneal endothelial cells maintain a delicate balance between hydration and dehydration in the corneal stroma essential for corneal clarity.[24] Corneal nerves are responsible for sensations of touch, pain, and temperature, controlling tear production, mediating the blink reflex, and impacting corneal wound healing.[25] Loss of neural innervation decreases epithelial cell proliferation and migration, which ultimately impairs epithelial function and wound repair.[26]

Herein, we present a 3D corneal construct with all 4 cell types predominant in the human cornea, assembled within a chemically crosslinked collagen matrix. The culture media was optimized to permit healthy growth of all 4 cell types. The corneal epithelial cells stratified appropriately on the anterior surface of the construct, and nerve cells grew within the construct to mimic native innervation. This 4-cell corneal construct represents a technical advance in the development of an in vitro 3D model of the human cornea and is potentially useful for the study of different disease conditions, molecular or drug interventions, and testing novel therapeutic approaches.

Methods

The protocol for use of cell lines initially derived from deceased human donors was approved by the Massachusetts Eye and Ear Human Studies Committee as exempt and is consistent with the principles expressed in the Declaration of Helsinki.

Cells and Media

SV40-immortalized human corneal epithelial cells (HCEC) and neuroblastoma cells (NDC) were used as previously described.[27,28] Telomerase-immortalized human corneal fibroblasts (HCF)[29] were kindly donated by James V. Jester, PhD, University of California. Telomerase-immortalized human corneal endothelial cells (CEC),[30] kindly provided by Ula Jurkunas, MD, Harvard Medical School.

Human corneal epithelial cells were grown in kKeratinocyte serum-free medium (SFM) (Life Technologies Corporation) supplemented with human recombinant epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 µg/ml). HCF were grown in DMEM/Ham's F-12 media supplemented with 10% fetal bovine serum (FBS) (Life Technologies). Corneal endothelial cells were grown in Opti-MEM I with Glutamax-I media (Life Technologies) supplemented with 8% (v/v) FBS, 5 ng/mL epidermal growth factor (EMD Millipore Corporation), 0.2 mg/mL calcium chloride (Fisher Scientific Company), 0.8 mg/mL chondroitin sulfate-A (Sigma-Aldrich), 0.25 mg/mL Gentamycin (Life Technologies), 1% (v/v) antibiotic-antimycotic solution (Life Technologies), and 0.1 mg/mL bovine pituitary extract (Thermo Scientific Chemicals). Cell culture surfaces were coated with the mixture of fibronectin, collagen, and albumin (Athena Environmental Sciences) before culturing CEC. Neuroblastoma cells grew in DMEM (Thermo Scientific) media supplemented with 10% FBS. All the media were supplemented with 1% penicillin and streptomycin (Gibco, Life Technologies Corporation).

Optimization of Cell Culture Media

To attain a normal phenotype for each of the 4 cell types within the same 3D construct, it was necessary to develop and optimize a single culture medium that would enable normal cell growth support to each cell type. After testing of various available media, DMEM/Ham's F-12 (Corning) with 10% newborn calf serum and 10 ng/ml epidermal growth factor was selected for the culture of the corneal construct. We refer to this as STRAT media for rest of this article. STRAT media had been previously evaluated for promoting stratification of corneal epithelial cells.[29,31] The effect of STRAT media on cell proliferation in comparison to each cell type's standard growth media was tested individually for all 4 cell types (HCEC, HCF, CEC, and NDC) with AlamarBlue at 1, 4, and 6 days after cell seeding. At each time point, the cell culture media was removed and replaced with fresh media containing resazurin sodium salt (0.004% w/v) and incubated for 4 h, at which time the media was removed and analyzed on a BioTek plate reader (Synergy 2, BioTek Instruments) at 530/25 nm for excitation and 600/25 nm for emission. At day 6 post seeding, live/dead staining was also performed (Life Technologies Corporation), in which cells were double-stained by calceinacetoxymethyl and ethidium homodimer-1 (EthD-1). Images were taken with a fluorescence microscope (Zeiss Axio Observer Z1, Carl Zeiss Microimaging GmbH).

Corneal Construct Fabrication

The construct was formed from collagen type I derived from rat tail (3−4 mg/ml) (Corning). Collagen buffer (10X HEPES:10X DMEM: FCS = 1:1:1.11) was added to collagen (3.3:1 v/v collagen: buffer) together with chondroitin sulfate (12:1 v/v collagen) and neutralized with 1N NaOH. The mixture was crosslinked with aqueous glutaraldehyde (0.022%, corresponding to the initial collagen volume) and unreacted aldehyde groups were neutralized by adding glycine solution (1.66%, corresponding to the initial collagen volume). Approximately, 1×10^5 HCF per ml were added to the mixture, and it was molded in a 0.3 µm pore sized, 12 mm diameter cell culture inserts (Coster) that had been coated with fibronectin, collagen, and albumin and seeded with 33×10^4 CEC in CEC growth media 1 day earlier. Then, 1×10^4 NDC were seeded per insert with equal density at 4 different points at the periphery of the insert. Approximately, 5×10^5 HCEC were seeded per insert on the top of the matrix. STRAT medium was added outside the insert to create an air-fluid interface and the total construct was maintained for 7 days.

Cell Viability

At day 7 of the culture, constructs were stained whole mount with calceinacetoxymethyl and EthD-1 (Thermo Fisher) and examined by confocal microscopy (Leica TCS SP8 Confocal Microscope). For selective staining and visualization of specific cell types, constructs were also generated with all cell types but NDC, all cell types but HCF, or only with CEC.

Histology

Constructs were fixed with 4% paraformaldehyde, dehydrated with increasing concentrations of ethanol (70%, 96%, 100%) for 120 min at each concentration, and paraffin-embedded by immersion in xylene and then in liquid paraffin overnight. Paraffin-embedded sections were cut with a microtome and stained with hematoxylin for 2 min and eosin for 1 min (H&E, Thermo Fisher), as previously published.[29]

Western Blot

Whole construct and control cell (HCEC, HCF, CEC, and NDC) lysates were prepared in lysis buffer and protease inhibitor cocktail (Cell Signaling). Equal aliquots of control cell and corneal construct lysate proteins were resolved on Novex WedgeWell 4% to 20% Tris-Glycine protein gels (Thermo Fischer). The proteins were transferred to nitrocellulose membranes (Bio-Rad), immunoblotted with primary antibodies (Supplementary Table 1, available at www.ophthalmologyscience.org), followed by a secondary antibody conjugated to horseradish peroxidase (Supplementary Table 1, available at www.ophthalmologyscience.org). The signals were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher) and imaged using a ChemiDoc imaging system (Bio-Rad).

Statistical Analysis

One-way analysis of variance with Tukey post hoc test was performed to compare groups. A value of $P < 0.05$ was considered statistically significant. ns, *, **, *** and **** represent nonsignificant ($P > 0.05$), $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. GraphPad Prism (GraphPad Software) was used to analyze the data. Data are shown as mean ± standard deviations.

Results

Cell Viability

Viability of all the cell types was evaluated in STRAT media and compared with respected maintenance media of individual cells (HCEC, HCF, NDC, and CEC). Live/dead staining (Fig 1A) showed that all cell types grew in the STRAT media, with very few dead cells observed. Proliferation studies performed at different time points (Fig 1B) demonstrated the viability and propagation of cells in both STRAT media and in cell specific maintenance media. Human corneal epithelial cells showed a higher proliferation rate on day 6 when cultured in KSFM media compared with the STRAT media; HCEC-KSFM versus HCEC-STRAT ($P < 0.0001$). However, a significantly high number of HCF were observed with STRAT media compared with maintenance media; HCF-DMEM/F12 versus HCF-STRAT ($P < 0.0001$). Similarly, NDC proliferation was also significantly higher with STRAT media: NDC-DMEM versus NDC-STRAT ($P < 0.0001$). Endothelial cell proliferation was comparable in both maintenance media (endo) and STRAT media: CEC-Endo versus CEC-STRAT ($P = 0.3583$).

Cell Viability in 3D Construct

In whole mount live/dead staining (Fig 2, B−E), green fluorescence indicates metabolic activity and live cells. EthD-1 stained red cells (dead) were not seen in any substantial number. Human corneal epithelial cells grew at the outer layer of the construct as a stratified layer. Both HCF and NDC were present below the HCEC within the collagen matrix. The Z-stacked images confirmed the presence of HCF in the same planes as that of NDC (Supplementary Fig. 2, available at www.ophthalmologyscience.org). Corneal epithelial cells were observed at the bottom of the construct as expected.

Histological Evaluation

The constructs were sufficiently stable to survive processing for histological evaluation (Fig 3) and could be easily removed from the culture insert (Fig 3A) for further processing by fixing and paraffin embedding (Fig 3B). H&E staining confirmed the formation of multilayered stratified epithelium on the top of the construct (Fig 3C). Scattered HCF (Fig 3D) and a monolayer of CEC (Fig 3E) were also visible upon H&E staining.

Cell Specific Protein Expression

Cell specific protein expression profiles within the corneal construct were probed with markers for HCEC, HCF, CEC, and NDC using immunoblot. The corneal epithelial markers cytokeratin 3 + 12 and MUC 16 (Fig 4A) were detected in both HCEC monolayer lysates used as a control, as well as in lysates of whole constructs where HCEC were grown as a stratified layer. Stromal cell specific ALDH3A1 (Fig 4B) was detected in both control HCF monolayers and in the constructs. Smooth muscle actin (SMA) was only detected in monolayer HCF cultures. Corneal endothelium specific marker ZO-1 (Fig 4C) was detected in both CEC control cultures and in construct lysates. Vimentin (Fig 4D) expression was more prominent in the corneal constructs than in control NDC cultures.

Discussion

A considerable amount of work has been done on the development and characterization of an in vitro corneal model capable of enabling detailed investigations into corneal biology and disease.[5] The current study further advances progress toward our goal of a bioengineered cornea-in-a-test-tube that would better predict in vivo responses and further reduce the need for animal studies of corneal diseases and treatments.

Neuroblastoma cells are a hybrid cell line derived from the fusion of neonatal rat dorsal root ganglia neurons and mouse neuroblastoma cells and has the potential to differentiate into neuron.[32,33] We grew NDC cells with medium supplemented with growth factors for their differentiation (see Supplementary Method, available at www.ophthalmologyscience.org), and the cells were stained with different neural markers. Our results were in line with previous findings that NDC were able to differentiate into mature neurons, expressing the neuron specific antigen neurofilament heavy chain, vimentin, and β-tubulin III (Supplementary Fig. 1, available at www.ophthalmologyscience.org) when cultured in STRAT medium. We note that collagen was used to coat the plates for NDC culture (see Supplementary Method, available at www.ophthalmologyscience.org) because our corneal construct was fabricated mostly with collagen type I extracellular matrix. Earlier studies using dorsal root ganglia from chicken embryos showed the

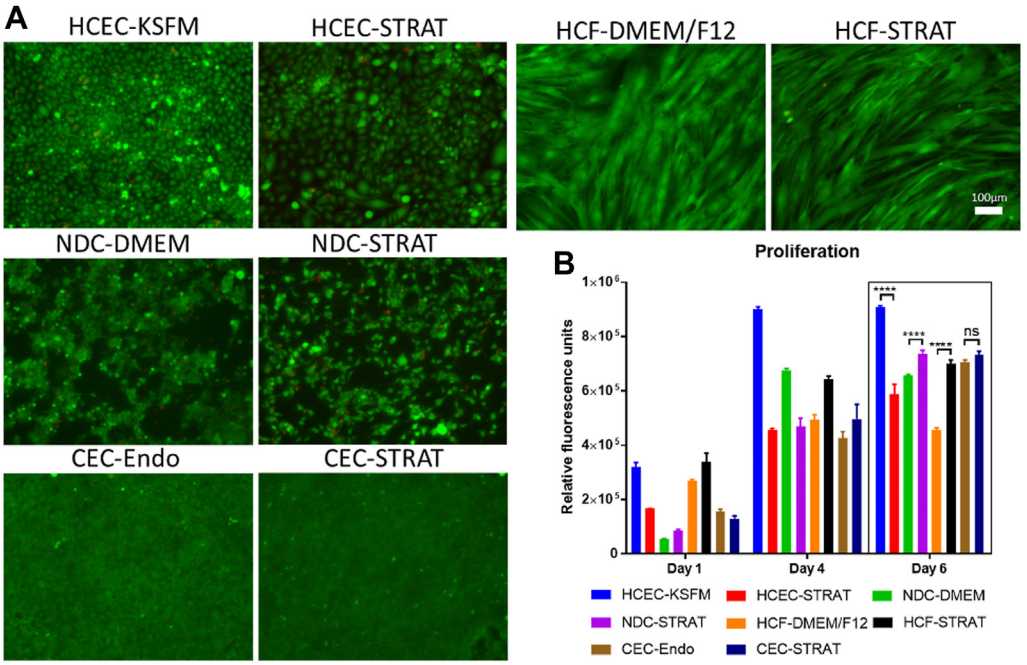

Figure 1. Comparative cell viability in the construct with STRAT media and in maintenance media. (**A**) Representative live/dead study images show that all the individual cell types survived in the STRAT media. Green and red colors indicate live and dead cells, respectively. Scale bar is 100 μm. (**B**) Comparative proliferation rates over time. Statistical analyses were performed on day 6. A value of $P > 0.05$ was considered statistically nonsignificant (ns). **** represents $P < 0.0001$. CEC = telomerase immortalized human corneal endothelial cells; HCEC = SV40-immortalized human corneal epithelial cells; HCF = immortalized human corneal fibroblasts; NDC = neuroblastoma cells.

importance of the presence of a nerve source and its influence on corneal epithelium after traumatic insult.[34] To the best of our knowledge, this work is the first time NDC cells have been used in an in vitro corneal construct together with the other 3 human corneal cell types.

Collagen was the main component of the corneal construct as the human cornea consists mostly of collagen, accounting for about 71% of the cornea stroma. Chondroitin sulfate was also used for the construct as glycosaminoglycans are the most abundant heteropolysaccharides in the human cornea.[35] Several previous studies have incorporated chondroitin sulfate into extracellular matrix to mimic the native cornea and increase the functional and mechanical properties of corneal constructs.[36] Glutaraldehyde has also been used successfully before in making corneal constructs.[7] Glutaraldehyde did not lead to toxicity because the unreacted aldehyde group was chemically neutralized by glycine.

We cocultured 4 different cell types in our 3D corneal construct. Each of these cell types has different and specific maintenance requirements. Therefore, developing a single coculture media that would facilitate growth of all 4 cell types was a necessity. Complex media concoctions

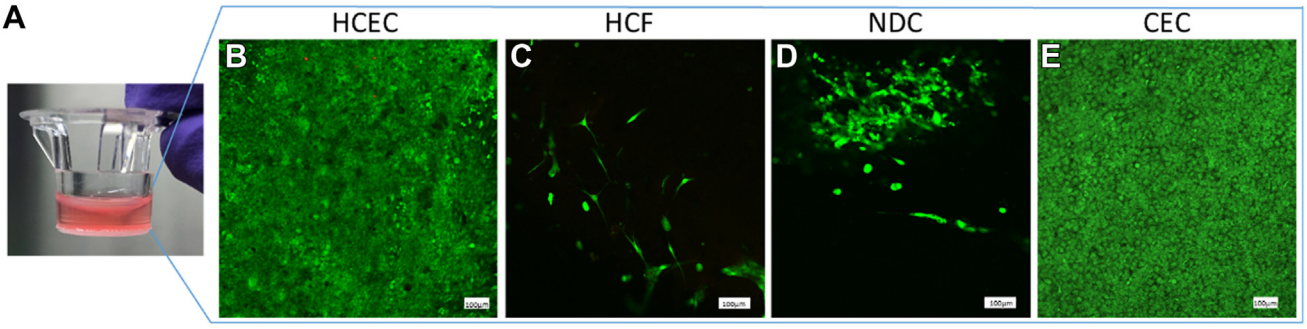

Figure 2. Corneal construct (**A**) within the transwell insert and live/dead staining images from top to bottom (**B**–**E**) of the whole mount construct. SV40-immortalized human corneal epithelial cells (HCEC) formed the topmost layer of cells (**B**), immortalized human corneal fibroblasts (HCF) and neuroblastoma cells (NDC) (**C** and **D**, respectively) were found in the middle collagen layer and telomerase-immortalized human corneal endothelial cells (CEC) on the bottom (**E**) of the construct. Green and red represent the live and dead cells, respectively. Scale bars are 100 μm. Perforations in the transwell membrane were also visible in the CEC image (**E**).

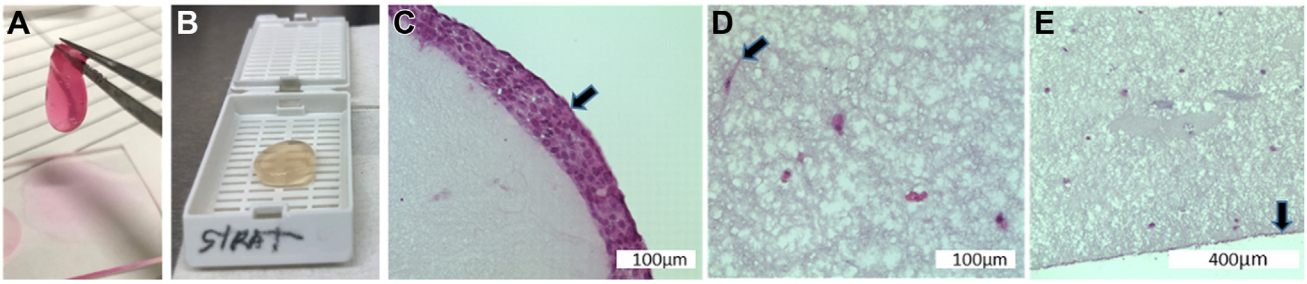

Figure 3. Histology of the corneal construct. (**A**) Full thickness corneal construct after removing from the insert. (**B**) Fixed and dehydrated construct before paraffin embedding. (**C**–**E**) hematoxylin and eosin (H&E) staining of the corneal construct shows specific cells at the expected layer. Arrows indicating stratified epithelium, stroma, and endothelium in the images of **C**, **D**, **E**, respectively.

including several supplemented ingredients have been used previously for corneal tissue models.[37,38] In the work described herein, we found and optimized a simple growth media for all 4 cell types used in the corneal construct. Unique among the principal corneal cell types, HCEC are typically grown in a serum free maintenance media. However, for stratification and differentiation, serum based media have been used to culture HCEC.[31] We previously showed that changing media from Keratinocyte SFM to STRAT facilitated the change from a confluent monolayer of HCEC to a 3 to 4 cell layer thick, stratified epithelium, as confirmed by H&E staining.[29] However, in past work, we only studied HCEC alone in STRAT media.[30] In the present work, we include 3 other cell types (HCFs, NDCs, and CECs) not tested earlier in STRAT media and found that STRAT media can facilitate the growth and maintenance of all 4 cell types within and on the constructs. Proliferation and cytotoxicity studies on cell monolayers confirmed that all 4 cell types grow in the STRAT media. Further, when grown on and within the corneal construct in the STRAT media, all 4 cell types survived and proliferated and were found to have normal cellular phenotypes as revealed by Western blotting. Further customization of the media by adding specific growth factors and/or other bioactive compounds could be used to study the biology of individual cell types in the setting of the 3D model.

Stratified epithelium is important for the protection and functional integrity of the normal human cornea. We generated an air-liquid interface to promote epithelial stratification, similar to prior investigations where an air-liquid interface promoted stratification of skin epithelial cells.[39,40] Minami et al, in 1993, first demonstrated that air-liquid interface also promotes corneal epithelial cell stratification on a collagen matrix.[13] In our in vitro construct, H&E staining confirmed the stratification of corneal epithelium, together with stromal cells in the matrix and single layer of endothelial cells at the bottom. Western blot analysis of

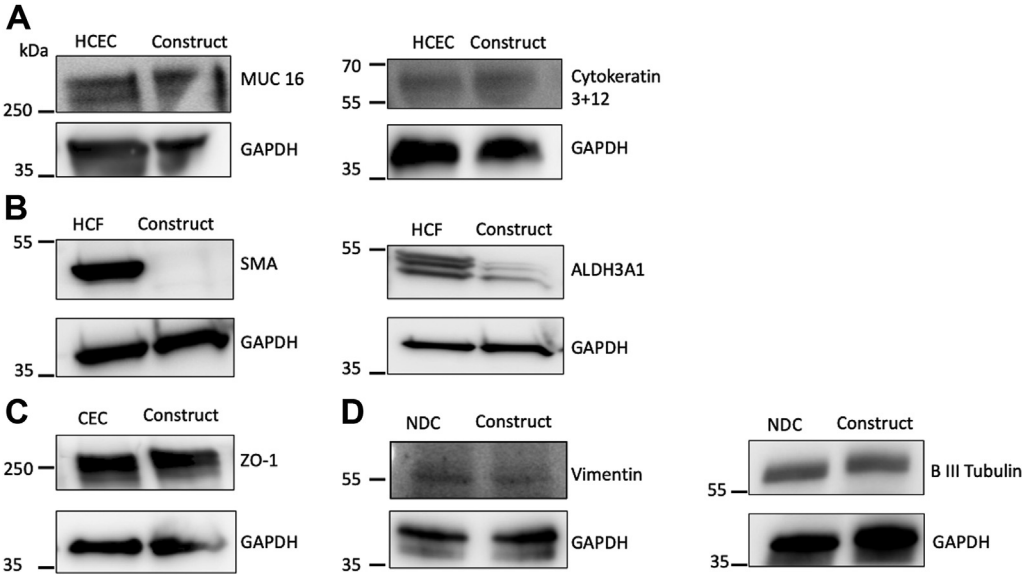

Figure 4. Immunoblotting of corneal construct. Western blot analysis of the expression of cell markers (MUC16 and cytokeratin 3 + 12 (**A**), Smooth muscle actin and ALDH3A1 (**B**), ZO-1 (**C**), and vimentin and β III tubulin (**D**) in corneal constructs were compared with positive control cell lines grown in monolayer cell culture (SV40-immortalized human corneal epithelial cells [HCEC], immortalized human corneal fibroblasts [HCF], telomerase-immortalized human corneal endothelial cells [CEC], or neuroblastoma cells [NDC], respectively).

whole corneal constructs confirmed cell specific protein expression by respective individual cell types, similar to the cell-type specific protein expression by the corresponding cell lines when cultured in monolayers. Although HCEC and CEC showed characteristic protein expression both in monolayer and in the corneal construct, only monolayer cultures of HCF expressed SMA. Smooth muscle actin expression is considered as an indicator of a myofibroblast phenotype,[41] and a prior study showed SMA expression by HCF when cultured in a collagen matrix.[42] Myofibroblast differentiation from HCF was previously shown to correlate with increased cell density and cell-cell contact,[41] perhaps explaining why HCF in our contrast retained a fibroblast phenotype. Alternatively, crosslinking the collagen matrix might have influenced the microenvironment for the HCF and led to retention of a fibroblast phenotype. ALDH3A1 is a keratocyte markers thought to indicate relative quiescence.[43] We expected to observe overexpression of ALDH3A1 but observed relatively less ALDH3A1 in constructs compared with monolayers. Note that, although we could not directly quantify the number of cells included in the corneal construct lysates because of the presence of collagen, the GAPDH levels in both lanes were similar for both blots, signifying a relatively equal number of cells in each sample. This observation followed our previous report in which we found limited ALDH3A1 expression when primary HCF were cultured on a collagen matrix in the presence of serum.[44] The keratan sulfate-containing proteoglycan keratocan is one of the phenotypic markers of healthy keratocytes and is essential for corneal transparency.[45] We have previously shown that modified biocompatible artificial corneal surfaces can support a physiologically normal expression of ALDH3A1, SMA, and keratocan by human corneal fibroblasts. Biocompatibility was evident by expression of ALDH3A1 and keratocan, with relatively lower expression of SMA.[46] In that published study, keratocan expression became evident after 45 days of cell culture. In the current study, we observed a similar pattern of ALDH3A1 and SMA expression, but we likely need to maintain the constructs in culture for longer to observe normal, physiologic expression of keratocan.

Vimentin is a filament protein expressed by different cells, including astrocytes and neural stem and progenitor cells.[47] Neuroblastoma cells cells showed expression of vimentin in tissue culture plate culture (Fig 3D) and the expression increased after culturing with differentiation media (Supplementary Fig. 1, available at www.ophthalmologyscience.org). Increased expression of vimentin was observed by Western blot on NDC cultured in corneal constructs, potentially because of the STRAT media. Further evaluation is required to understand vimentin expression by NDC in the corneal construct.

Development of a stable corneal construct was the main goal of this project, but we have not yet performed functionality studies with the construct. As recently summarized by Kaluzhny and Klasuner,[48] Griffith et al was one of the first to generate an in vitro corneal model comprised of human cells,[5] showing that an in vitro cornea model could mimic the human cornea in morphology, biochemical marker expression, transparency, ion and fluid transport, and gene expression. In another study, biodegradable corneal constructs made from crosslinked collagen and implanted into pig corneas as lamellar grafts showed evidence for corneal nerve ingrowth and regeneration.[49] However, ours is the first to incorporate neuronal cells into a corneal construct in vitro.

In summary, we report the development of an in vitro corneal construct with all 3 major corneal cell types together with a source of neuronal cells. Our model can be a useful tool for studying corneal innervation and cell−cell interactions in a controlled environment and may permit the reduced need for animals in corneal research. The current construct cannot be transplanted into the human eye because of the use of continuous cell lines and its relatively insufficient mechanical rigidity. With further modifications to the extracellular matrix and the use of primary cells derived from the recipient, this approach could be applied in the future for corneal replacement. In this work, our focus was to make an in vitro construct of the cornea. However, a similar approach could be implemented to develop other organ-specific bioengineered substitutes for testing disease conditions and evaluate the effectiveness of potential therapies.

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Footnotes and Disclosures

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Analysis and interpretation: Islam, Saha, Trisha, Gonzalez-Andrades, Patra, Griffith, Chodosh, Rajaiya

Obtained funding: Islam, Chodosh, Rajaiya

Overall responsibility: Islam, Saha, Trisha, Gonzalez-Andrades, Patra, Griffith, Chodosh, Rajaiya

Abbreviations and Acronyms:
3D = 3-dimensional; **CEC** = telomerase immortalized human corneal endothelial cells; **EthD-1** = ethidium homodimer-1; **FBS** = fetal bovine serum; **H&E** = hematoxylin and eosin; **HCEC** = SV40-immortalized human corneal epithelial cells; **HCF** = immortalized human corneal fibroblasts; **NDC** = neuroblastoma cells; **SFM** = serum free medium; **SMA** = smooth muscle actin.

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An in vitro 3-dimensional Collagen-based Corneal Construct with Innervation Using Human Corneal Cell Lines

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Corneal research largely depends on animal models. To reduce animal use and improve translation of *in vitro* data to the clinic, we developed a three-dimensional corneal model which closely mimics the human cornea.