

The Development of a Tissue-Engineered Cornea: Biomaterials and Culture Methods

AJAY SHAH, JAMIE BRUGNANO, STACY SUN, AJOY VASE, AND ELIZABETH ORWIN

Division of Health Sciences & Technology [A.S.], Harvard-MIT, Cambridge, Massachusetts 02139; Department of Biomedical Engineering [J.B.], Purdue University, West Lafayette, Indiana 47907; Department of Engineering [S.S., E.O.], Harvey Mudd College, Claremont, California 91711; Department of Physics [A.V.], Pomona College, Claremont, California 91711

ABSTRACT: The field of corneal tissue engineering has made many strides in recent years. The challenges of engineering a biocompatible, mechanically stable, and optically transparent tissue are significant. To overcome these challenges, researchers have adopted two basic approaches: cell-based strategies for manipulating cells to create their own extracellular matrix, and scaffold-based strategies for providing strong and transparent matrices upon which to grow cells. Both strategies have met with some degree of success. In addition, recent advances have been made in innervating a tissue-engineered construct. Future work will need to focus on further improving mechanical stability of engineered constructs as well as improving the host response to implantation. (*Pediatr Res* 63: 535–544, 2008)

The cornea is the protective window of the eye, providing 75% of the eye's refractive power and transmitting 90% of blue and 98% of red light (1). Over 10 million individuals worldwide experience bilateral corneal blindness (2). Corneal disease is a major cause of blindness, second only to cataracts (3). Corneal transplants are currently the only treatment for restoring vision. Total corneal transplantation has a 90% success rate in patients with good prognoses (low graft vascularization and inflammation), but almost no chance of success in patients with alkali burns or recurrent graft failures (4). The shortcomings of corneal transplantation include significant immune rejection rates (5,6), the possibility of infections (7,8), and donor shortages (7). A tissue-engineered corneal replacement could provide significant benefits as an alternative to donated corneas.

The cornea consists of three layers: the innermost endothelial cell layer, the stroma, and the outermost epithelium, which is in direct contact with the environment. The epithelium is regenerative and provides protection for the surface of the eye (9). The stroma, which makes up 90% of the cornea's 500- μ m thickness, consists of a pseudocrystalline lattice of collagen fibers, proteoglycans, and sparsely populated quiescent stromal cells (keratocytes) (10). The innermost layer of the cornea, the endothelium, is a single cell layer that provides a leaky barrier with high metabolic activity (11) and maintains corneal transparency by regulating stromal hydration using ATPase pumps (12). The cornea is a highly innervated tissue,

which is known to be important for epithelial cell health and function, as well as for corneal wound healing (13).

Constructing a cornea presents two major challenges to the field of tissue engineering: tissue strength and transparency. These properties are a result of the unique structure of this tissue, which is difficult to replicate. Corneal transparency depends on both tissue structure and cellular protein expression. The orderly array of collagen fibers and the refractive index matching of these fibrils by interstitial proteoglycans play a significant role in the transparency of the cornea. Each collagen type I fiber in the stroma is 20–35 nm in diameter. The fibers are aligned parallel to each other with regular 30-nm spacing between fibrils. This regular spacing is thought to be regulated by the proteoglycans, which have been observed to form ring-like structures around collagen fibrils in the normal cornea (14). The aligned fibers are grouped into layers called lamellae and stacked in an alternating lattice pattern (15). The thickness of the stroma and arrangement of the collagen fibers are optimal for visible light waves to pass through the cornea with minimal light scatter.

There is also a cellular contribution to corneal transparency, making the characterization of the phenotype of corneal keratocytes in normal and wounded corneas the subject of much investigation. Keratocytes express certain proteins, known as corneal crystallins, which are thought to match the refractive index of the cell cytoplasm with the surrounding matrix material (15). Stromal wounding in response to damage or surgical procedures initiates a distinct response in corneal keratocytes (9). Keratocytes differentiate first into repair fibroblasts, which can actively proliferate and synthesize new extracellular matrix (ECM) components. These repair fibroblasts can further differentiate into myofibroblasts, which express different proteins and are highly light scattering. Differentiation to myofibroblasts is reversible upon completion of the wound-healing process. However, if the repair fibroblast differentiates into a scar keratocyte, the process is irreversible and results in the formation of a corneal scar. The three

Abbreviations: α -SMA, α -smooth muscle actin; ALDH1, aldehyde dehydrogenase 1; AM, amniotic membrane; DRG, dorsal root ganglia; EGF, epidermal growth factor; ECM, extracellular matrix; PDMS, polydimethylsiloxane; PGA, poly-glycolic acid; TERP, [poly(*N*-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide)], PNiPAAm-coAAc-coASI; TKT, transketolase; UTS, ultimate tensile strength; UV, ultraviolet

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Correspondence: Elizabeth J. Orwin, Ph.D., Department of Engineering, Harvey Mudd College, 301 Platt Blvd., Claremont, CA 91711; e-mail: orwin@hmc.edu

phenotypically distinct cell types (keratocytes, repair fibroblasts, myofibroblasts) each express different proteins. In quiescent rabbit keratocytes, 30% by mass of the water-soluble protein is comprised of the “corneal crystallins” transketolase (TKT) and aldehyde dehydrogenase 1 (ALDH1) (15). When corneal haze is present following injury (and therefore activation of keratocytes to the repair or myofibroblast phenotype), there is a 50% reduction in expression levels of these proteins (15). It is hypothesized from these results that TKT and ALDH1 act similarly to crystallin proteins in the lens of the eye – that is, they accumulate in the cell cytoplasm and contribute to index matching of the cytosol with the surrounding extracellular environment. Jester and colleagues have shown that for normal keratocytes in a transparent cornea, light scatters only from the nucleus of the cell but not from the cell body, as further evidence for this theory (15). The corneal myofibroblast phenotype present during wound healing is characterized by the expression of α -smooth muscle actin (α -SMA), a filamentous protein that plays a role in the matrix contraction (9). Cultured rabbit corneal myofibroblasts scatter more light than keratocytes and exhibit reduced expression of both TKT and ALDH1 (16). A recent study in human cells corroborates the rabbit studies: ALDH3 and TKT are both reduced in repair fibroblast and myofibroblast populations compared with the quiescent keratocyte (17).

The challenges to creating a tissue-engineered cornea include creating a construct that is cytocompatible, mechanically strong, and optically transparent. Recent advances in corneal tissue engineering have attempted to use the discoveries about both matrix structure and cellular behavior to their advantage. Accordingly, two broad categories of approaches to this problem have emerged: cell-based approaches and matrix-based approaches. In this paper, we will summarize the recent literature in both of these areas. In addition, we will address the current advances in creating a full tissue-engineered cornea, as well as the challenges that remain in this field.

CELL-BASED APPROACHES TO A TISSUE-ENGINEERED CORNEA

Some studies have investigated cell-based methods to take advantage of the information contained in cells themselves to mimic the structure and function of the native human cornea. To recreate the corneal stroma, fibroblasts have been manipulated to synthesize their own ECM. Epithelial and endothelial cells have been engineered as complete cell sheets using a novel method that utilizes specific polymer properties to alter hydrophobicity in response to temperature. Other studies have attempted to engineer a complete cornea by co-culturing two or more of the corneal cell types.

Fibroblast culture methods. During corneal development, the collagen fibrils are regularly spaced and precisely aligned – a structure which contributes to optical transparency. The extent to which stromal cells manipulate and interact with their ECM is being investigated to engineer a stratified stromal layer with a supporting matrix. Studies have shown that ascorbic acid can increase fibroblast proliferation and stimulate collagen secretion from the stromal cells (18–20). Recently, human kerato-

cytes, when cultured in a stable form of ascorbic acid, have been shown to assemble a three-dimensional matrix comprised of parallel collagen fibers similar to those found in the human cornea (21). By the fourth week in culture, fibroblasts formed a construct with alternating arrays of fibrils with a total thickness of $36.3 \pm 6.6 \mu\text{m}$, and a structure similar to that of the native corneal stroma (Fig. 1). Since the average difference in the measured angle between the cell axes and the fibril orientation was minimal ($0.65^\circ \pm 0.49^\circ$), the fibroblasts were considered to be aligned with their corresponding fibrils. High cell density, the presence of rough endoplasmic reticulum, and fibril clusters further indicated a high level of similarity to the native corneal stroma. However, the average fiber diameter ($38.1 \pm 7.4 \text{ nm}$) was thicker than that of the natural corneal stroma ($30.1 \pm 2.5 \text{ nm}$), suggesting a need to further investigate cell stimuli to reduce the fibril diameter size. These studies show the promise of harnessing cells to self-assemble a corneal matrix; however, further studies are necessary to determine the limits of thickness of such a construct, as well as to assess mechanical and optical properties of the tissue.

Another recent study looked at cell morphology and collagen production from stromal fibroblasts grown on collagen matrices with different mechanical properties (22). Cells were grown on matrices constrained along a single axis and compared with unconstrained controls. This study found that corneal fibroblasts seeded on collagen matrices align along the axis of ECM stiffness and produce more collagen, which is also aligned along the axis of ECM stiffness. These results suggest that mechanical signaling could induce corneal stromal cells to produce an aligned collagen matrix approximating that of the native cornea. Another recent study discusses material selection for a bioreactor designed to apply appropriate mechanical signals to developing corneal tissue (23). These studies indicate the growing interest in mechanical stimuli for corneal tissue development.

Epithelial cell culture methods. Several strategies have been developed to create transplantable epithelial cell sheets for the treatment of ocular surface disease. These cell sheets have direct applicability to tissue-engineered corneas in that

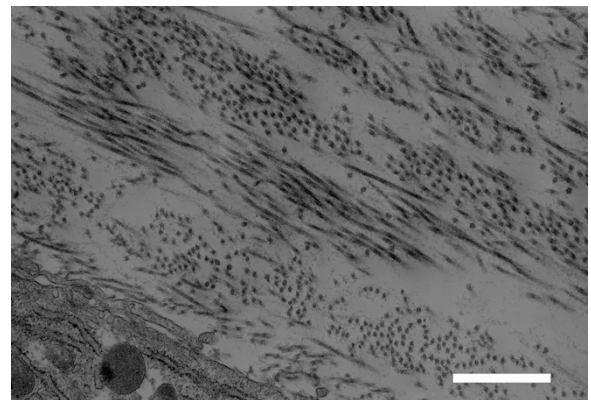


Figure 1. Transmission electron micrographs of collagen fibers secreted by human stromal fibroblasts stimulated by ascorbic acid *in vitro*. The fibers are aligned within an alternating array construct, similar to the native corneal matrix orientation. Image reprinted from Guo *et al.*, Invest Ophthalmol Vis Sci 48:4050–4060, Copyright © 2007, The Association for Research in Vision and Ophthalmology, with permission.

they can be used to form the epithelial and endothelial surfaces of the cornea after a stroma has been developed. In the case of the epithelium, cell sheets can easily be created from autologous cells using a small biopsy of native tissue. The two most promising approaches for engineering epithelial cell sheets are the use of temperature-responsive polymer surfaces and biodegradable fibrin layers.

By utilizing a novel approach with temperature-responsive culture dishes, epithelial cell sheets have been engineered and transplanted directly on the eye without sutures. At 37°C, the temperature-responsive polymer, poly(N-isopropylacrylamide), is hydrophobic and allows for cell attachment and proliferation. However, by reducing the temperature to 20°C, the polymer becomes hydrophilic and expands rapidly, resulting in cell detachment. Thus, cultured cells can be harvested as intact sheets along with their deposited ECM in contrast to proteolytic harvest methods that break up the ECM and crucial cell-cell interactions. Epithelial cell sheets demonstrate a compact, stratified structure that resembles a native epithelial cell sheet (24). Because the polymer remains covalently attached to the dish, it remains separate from the epithelial cell sheet (24). During transplantation, the epithelial cell sheet attaches spontaneously to the ECM and adhesive proteins of the host cells (24,25). Hayashida *et al.* cultured epithelial cells, which stratified into a thick sheet with three to five differentiated cell layers, on these temperature-responsive culture dishes and transplanted the cell sheets into rabbit eyes that had undergone excimer laser photoablation. In rabbits with the transplanted cell sheet, all epithelial defects from the laser ablation healed immediately and had little to no evidence of epithelial hyperplasia. In contrast to the subjects without transplantation, the transplanted subjects experienced a significant decrease in corneal haze as demonstrated by the diminished expression of collagen III and α -SMA in all areas of the stroma (25).

An alternative method to temperature responsive polymer surfaces was reported by Higa *et al.* This group seeded corneal epithelial cells onto a fibrin sealant that was later digested to create a stratified epithelial sheet consisting of five to six layers of cells (26). The cell sheets expressed the epithelial differentiation markers K3 and K12, and demonstrated tight junction formations and adhesive proteins. After culture, the corneal epithelial cell sheets were detached with a cell scraper and transplanted onto a rabbit's bare corneal stroma without sutures. In comparison with the epithelial sheets cultivated on amniotic membrane carriers, the epithelial sheets engineered with the fibrin sealant were more differentiated and retained a similar level of colony-forming efficiency.

Endothelial cell culture methods. Similarly, corneal endothelial cell sheet engineering has been achieved from temperature-responsive culture surfaces. Studies have demonstrated that cell morphology, cellular interconnections, and the monolayer architecture of endothelial sheets harvested from thermoresponsive culture mimic the natural corneal endothelium (27,28). Sumide *et al.* determined that the number of sodium-potassium ATPase pump sites ($4.65 \pm 0.2 \times 10^9$ pump sites/mm²) were similar to those of the native corneal endothelium ($4.4 \pm 0.2 \times 10^9$ pump sites/mm²). In comparison to control rabbit eyes that did not receive the endothelial trans-

plant, the transplant recipients experienced overall reduced corneal swelling (transplants: $496 \pm 111.6 \mu\text{m}$, controls: $887.5 \pm 69.0 \mu\text{m}$). Slit lamp microscopy demonstrated that, in comparison with the control group, the corneas with the endothelial cell sheet had increased corneal transparency. Lai *et al.* also engineered endothelial cell sheets using the temperature-responsive technique and demonstrated that viable endothelial cell sheets with intact barrier and pump functions can be obtained without the need for cell carriers during cultivation (27).

Co-culture methods. In addition to monoculture, studies have co-cultured two types of corneal cells together to investigate cell-cell interactions in an attempt to better stimulate normal cell behavior. Epithelial cells have been co-cultured with fibroblasts on collagen gels (29). Gels with injured epithelial cells demonstrated greater gel contraction, cell density, and α -SMA levels than gels with uninjured or without epithelial cells. This suggests that injured epithelial cells secrete a soluble factor, which stimulated collagen gel contraction by fibroblasts, proliferation of fibroblasts, and myodifferentiation of fibroblasts (29). Epithelial cells co-cultured with fibroblasts on collagen sponges have also been shown to form a continuous layer of cells on top of the sponge surface that exhibits the columnar morphology similar to that of the native basal corneal epithelium (30). Similarly, epithelial cells seeded on a collagen sponge and co-cultured with or separated from endothelial cells by a transwell culture insert formed three to four layers of epithelial cells by the second week in culture, compared with the single layer of epithelial cells observed when cultured on collagen alone (30). Zieske *et al.* co-cultured all three corneal cell types together in a three-dimensional tissue construct to investigate the effect of culture conditions and endothelial cell interaction on epithelial cell differentiation and basement membrane assembly (31). A basal layer of endothelial cells was covered with a middle layer of stromal fibroblasts in a collagen gel matrix and covered with a top layer of epithelial cells. Including endothelial cells in the construct resulted in the epithelial cells forming a basal layer with columnar morphology and stratified layers. In addition, laminin and collagen type VII became localized as continuous bands at the epithelial-matrix junction. These results indicate that the presence of endothelial cells induces the assembly of an epithelial basement membrane and differentiation similar to a native corneal epithelial cell layer.

SCAFFOLD-BASED APPROACHES: MATERIALS USED FOR TISSUE-ENGINEERING A CORNEAL STROMA

The other major approach in corneal tissue engineering involves the design of a novel substrate on which to grow the cells. A variety of different biomaterials have been used for corneal tissue engineering. These biomaterials must be optically transparent, biocompatible, mechanically stable, and allow cells to adhere, proliferate, and migrate. Many investigators have focused on scaffolds made up of type I collagen to mimic the composition of the native cornea. Others have attempted to use synthetic polymeric based approaches to

better mimic the mechanical properties of the cornea. This review will focus on the recent progress of scaffolds for corneal equivalents but earlier reviews can be found (32).

Collagen gel-based approaches. Type I collagen gels have been used in a number of studies. One group used primary culture corneal cells and a cell-perfusion culture method in a collagen gel model (33). Cells were isolated from the corneas of chick embryos and rabbits. The scaffold consisted of an alkaline solubilized collagen gel crosslinked with pentaerythritol polyethyleneglycol ether tetrasuccinimidyl glutarate. The gel was placed in a ring shaped apparatus with polyvinylidene difluoride membrane support to provide static stress during the gel shrinkage process and cultured for a maximum of 60 days. To maintain oxygen and nutrient supply, the gel was set in a container-chamber and cultured under perfusing media. The cells increased in number within and on the surface of the gels; however, transmission electron microscopy showed no collagen fibril formation after 11 days. This indicates that the function of the stroma was not replicated completely, although the cellular morphology was similar to that of the native cornea. The transparency of the gel was sufficient to conclude that this technique has the potential to produce biocompatible stromal equivalents; however, further investigations into the biomechanical properties of this stromal equivalent are still needed.

Collagen gels have poor mechanical properties and degrade rapidly *in vivo*. Investigators have attempted to improve the mechanical properties of collagen gels by exploring different crosslinking methods. Doillon *et al.* investigated the properties of Type I collagen (0.3 wt/vol %) combined with chondroitin sulfate (CS) (1.2% vol/vol) crosslinked with various concentrations of glutaraldehyde (0–0.08%) and posttreated with a glycine solution to minimize the cytotoxic effects of glutaraldehyde (34). Glutaraldehyde concentrations up to 0.04% strengthened the matrix and allowed cell growth with the optimum glutaraldehyde concentration of 0.02%. Chondroitin sulfate types A, B, and C were added to the collagen gel matrices and optimal results were found with the addition of form C. Chondroitin sulfate C improved the transparency of the matrix from 55% light transmission without CS to 66% light transmission with form C. Duan and Sheardown introduced polypropyleneimine octamine dendrimers as crosslinkers of concentrated collagen gels (35). This crosslinker increases the number of amines that are available for reaction with activated carboxylic groups for crosslinking. Properties of the dendrimer crosslinked collagen gels were compared with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and glutaraldehyde crosslinked collagen gels. Mechanical properties of the novel crosslinked gel were higher than EDC and glutaraldehyde crosslinked samples with a Young's modulus ranging from 1.4 to 5.3 MPa based on different collagen concentrations. This is in the range of the modulus of natural human cornea (0.3–7 MPa). The strength at maximum load was reported to be 1.2 ± 0.7 N, which is much lower than that of the cornea. The suture strength of the gels, however, was much lower than that of the native cornea. The dendrimer scaffold had high transparency measured *via* spectrophotometry (80% transmittance at 400 nm and up to

95% transmittance at 700 nm) and allowed the growth and adhesion of immortalized human epithelial cells. However, corneal fibroblasts have not been cultured on the construct, nor is it clear if cells can migrate through the scaffold.

Other collagen configurations (sponges/films). Orwin *et al.* investigated the biomechanical and optical characteristics of a stromal equivalent built on a collagen sponge matrix with human corneal stromal cells (36). The sponges used in the study were prepared from bovine type I dermal collagen by blending ground collagen with H₂O + HCl at 4°C to form a dispersion, deaerating and lyophilizing at –30°C. Sponges were dehydrothermally (DHT) crosslinked and sterilized before cell seeding. Collagen gels were prepared from Vitrogen 100 (Cohesion Technologies, Palo Alto, CA) and used as controls. Unconfined stress-relaxation tests were performed on the stromal equivalent. A compressive strain of 15% was applied to the sample and the force exerted by the sample was recorded. The modulus for the gel (11.6 ± 14.7 Pa at day 14 in culture) was an order of magnitude less than the sponge (177 ± 75 Pa at day 21 in culture). The study found that the fraction of light transmitted through the gels (0.05) is statistically significantly less than that transmitted through the sponges (0.33) at 700 nm and 21 d in culture. In addition, collagen sponges augmented with chondroitin sulfate transmitted ten times more light than collagen gels (0.57), and achieved more than 50% of the light transmitted by a freshly excised cornea. Nearly 100% of cells seeded at medium or high densities expressed α -SMA by day 21 in culture in a collagen sponge. The myofibroblastic phenotype of the cells within the sponge matrix may be contributing to the light transmission properties. This study shows that the collagen sponge model is an improvement over collagen gels both in mechanical properties and in tissue transparency. A follow-up study by this group further characterized cellular behavior and mechanical properties in a collagen sponge-based model over time in culture (37). The modulus decreased from 359 ± 57 Pa to 115 ± 73 Pa within the first two days of culture and then rose steadily thereafter. The permeability increased by four orders of magnitude during the first two days of compaction and then decreased slowly over the remainder of the experiment. These changes were affected by the compaction of the sponge, cell migration and proliferation, and degradation of the matrix. The study also found expression of fibronectin, decorin sulfate, collagenase and gelatinase, which suggest that cells were expressing the repair fibroblast phenotype. There was also an increase in the level of the α_5 integrin and a decrease in the level of the α_4 integrin expression, which indicates that a subpopulation of cells expressed the myofibroblast phenotype. This study highlights the relationship between the properties of the cells and matrix on a macro scale and cell behavior at the molecular level.

Collagen films have also been investigated as potential scaffolds for corneal replacement (38). Collagen films were prepared from dispersions of bovine type I collagen. DHT crosslinked films were shown to support the growth of stromal fibroblasts; however, fibroblasts did not migrate into the film. Collagen fibers within the films (233 nm) were significantly larger than what is found within the cornea; however, fibro-

blasts synthesized ECM fibrils 60–70 nm in diameter while growing on the films and approximately 100-nm fibers while growing in collagen sponges (Fig. 2). Cells on both substrates synthesized smaller fibers over 3 wk in culture. In addition, mechanical properties of collagen films were superior to collagen sponges, with relaxed moduli of 2.1 MPa for films *versus* 0.03 MPa for sponges. Cells responded to the collagen sponges by increasing α -SMA over time in culture, whereas phenotypic changes for cells on films were less distinct. This study indicates that the microstructure of the culture substrate can have an effect on cellular response. A follow-up study by this group (Crabb, 38a) demonstrated methods for improving the collagen film substrate in terms of mechanical and optical properties. Films produced from a mixture of insoluble collagen and soluble tropocollagen and crosslinked *via* a glucose-mediated ultraviolet (UV) method exhibited enhanced mechanical and optical properties. The relaxed modulus of the UV crosslinked films was higher (5.2 ± 0.3 MPa) than that for DHT crosslinked films (3.1 ± 0.3 MPa). These values are on the lower end of the reported values for the native cornea. Ultimate tensile strength (UTS) values for both types of films were on the order of 1.5 MPa, which is lower than the reported 19.1 MPa for the UTS of the native cornea (39) but much higher than the UTS of other collagen-based stromal equivalents. In addition, light transmission through the films matched that of the native cornea: 0.91 ± 0.02 for 400 nm light and 0.98 ± 0.01 for 700 nm light. The new film scaffold was stronger and more transparent than previous versions and other collagen configurations.

Augmented collagen. In an attempt to more accurately replicate the corneal stroma, several groups have augmented the collagen matrix with proteoglycans and other polysaccharides, and have produced aligned collagen substrates. Chen *et al.* used a collagen-chitosan-sodium hyaluronate complex and investigated the effects of varying the concentrations of each component on the growth of corneal epithelial cells, keratocytes, and endothelial cells (40). The optimal complex was made of 20% collagen, 10% chitosan and 0.5% sodium hyaluronate. Transparency was measured using UV absorption spectrum equipment and was found to have 95% light transmittance. This scaffold supported the growth of all three corneal cell types and degraded over a period of 5 mo when implanted in New Zealand albino rabbits. A minor inflammatory response was observed. Zhong *et al.* developed a collagen

and chondroitin sulfate (4 wt%) scaffold by electrospinning and crosslinking with glutaraldehyde vapor (41). Crosslinked scaffolds were found to have higher cell proliferation of rabbit fibroblasts compared with uncrosslinked scaffolds as determined by MTT assay. Electrospinning formed uniform nanofibrous and porous structures that are similar to the native cornea; however, the fibers were not aligned. Fiber diameter appeared to be on the order of hundreds of nanometers and transparency of the scaffold still needs to be measured. Torbet *et al.* achieved fibril alignment by using magnetic orientation to create a stromal like scaffold of orthogonal layers of oriented collagen type I fibers (42). Proteoglycans were added to improve the transparency of the matrix (35% decorin and 65% lumican, keratocan, and osteoglycin) and were found to reduce the size and variability of the collagen fibers. Layers were achieved by repeating the assembly process in a magnetic field (Fig. 3). Alignment was found to be achieved in as little as 5 min and keratocytes were found to align along the oriented fibers. Further studies will need to analyze the mechanical properties and crosslinking densities of these scaffolds.

Synthetic polymeric approaches. Some studies have also investigated using synthetic polymers to create a successful scaffold for a corneal equivalent. These scaffolds address some of the limitations of collagen; they do not shrink in the presence of cells and have more stable and tunable degradation properties. Zorlutuna *et al.* investigated the possibility of using natural polyesters, which are biodegradable and biocompatible (43). Poly(l-lactide-co-d,l-lactide) (70/30) and poly(hydroxybutyric acid-co-3-hydroxyvaleric acid) were evaluated for use as films for epithelial cells and foams for fibroblast cells. Adhesion of D407 epithelial cells and 3T3 fibroblasts was enhanced by fibronectin coating and functionality of the cell types were confirmed by immunohistochemistry. Foam thickness created a problem with fibroblast maintenance at the core of the scaffold; so further studies aimed at improving the porosity or decreasing the thickness of the foam need to be investigated. Mechanical properties of the polyester films have also been examined (44). Films were prepared by solvent casting blends of poly(l-lactide-co-d,l-lactide) (70/30) and poly(hydroxybutyric acid-co-3-hydroxyvaleric acid) on a micropatterned silicon template and seeding with human keratocytes or retinal pigment epithelial cells. Mechanical properties differed depending on the extent of cell coverage, with higher UTS observed on films that were covered in cells. Keratocytes showed an increase in tensile strength from 0.65 N/mm on day 7 to 0.73 N/mm on day 21 while epithelial cells showed a decrease from 0.98 N/mm on day 7 to 0.77 N/mm on day 21. A co-culture of these cells will be investigated in the future (44).

Klenkler *et al.* modified polydimethylsiloxane (PDMS) surfaces with epidermal growth factor (EGF) to improve adhesion of epithelial cells to the material surface (45). PDMS is cytocompatible, transparent, oxygen permeable and has appropriate mechanical properties. EGF was immobilized *via* a homobifunctional poly(ethylene glycol) spacer to animated silicon rubber surfaces to improve epithelialization in artificial cornea applications. EGF was shown to be active and stimu-

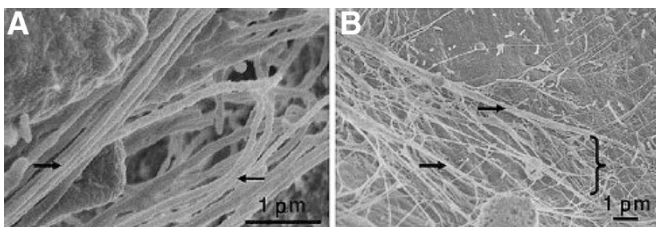


Figure 2. SEM images of extracellular matrix produced by fibroblasts grown on a collagen sponge matrix. The diameter of the produced fibrils decreases over time in culture from about 107 ± 20 nm at day 1 (A) to 47.5 ± 17 nm after 1 wk (B). Image adapted from Crabb *et al.*, Ann Biomed Eng 34:1615–1627, Copyright © 2006 Springer Science and Business Media, Inc., with permission.

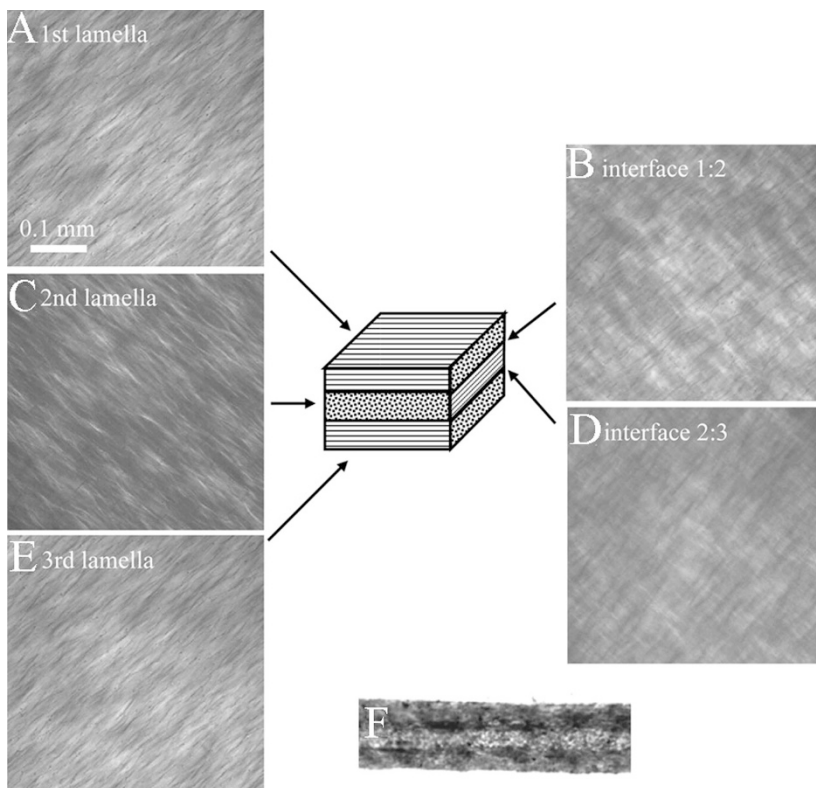


Figure 3. Optical micrographs using a polarizing microscope descending vertically through a ~ 2 mg/mL gel consisting of three (each ~ 0.5 mm thick) lamellae (A–E). Images show alignment within each layer and changes in orientation between layers, which is similar to the array of fibers in the native cornea. (F) Optical micrograph of three lamellae scaffold seen in cross section perpendicular to the lamellae surface where the middle layer is perpendicular to the other two. Image reprinted from Torbet *et al.*, *Biomaterials* 28: 4268–4276, Copyright © 2007, Elsevier Ltd., with permission.

lated both adhesion and growth of epithelial cells. Future work will address the effect of EGF on the production of ECM proteins. PDMS surfaces were also modified with transforming growth factor $\beta 2$ (TGF $\beta 2$) to stimulate stromal proliferation while inhibiting the proliferation of corneal epithelial cells (46). Contrary to expected results, corneal stromal cells exhibited very low adhesion to TGF $\beta 2$ coated surfaces (3–5%) while epithelial cells exhibited high levels of adhesion (50–70%) after one week of culture. Higher concentrations of TGF $\beta 2$ resulted in greater cell adhesion for both cell types. It is unclear why corneal stromal cells were unable to adhere to the PDMS surface; stromal cells might require additional growth factors, adhesion peptide incorporation on the surface, or co-culture with epithelial cells to successfully adhere.

Preventing protein adsorption can increase transparency and biocompatibility as protein adsorption has been postulated to trigger calcification, inflammation and retroprosthetic membrane formation. To this end, Myung *et al.* fabricated a hydrogel composed of a poly(ethylene glycol)/poly(acrylic acid) core with type I collagen tethered to the surface *via* the heterobifunctional crosslinker, 5-azidonitrobenzoyloxy *N*-hydroxysuccinimide (47). Surrounding the core is a microperforated poly(hydroxyethyl acrylate) hydrogel that is also modified with type I collagen. The resulting material is highly transparent (96%) with a refractive index of 1.35, which is slightly lower than that of the cornea, 1.376, and a high tensile strength up to 470 kPa. Modification of the surface with

collagen type I allowed epithelial cells and stromal cells to adhere and grow on the core and poly(hydroxyethyl acrylate) skirt, respectively. Previous studies by this group have shown that the hydrogels remain transparent in rabbit corneas up to 6 weeks (Bakri A *et al.* ARVO Annual Meeting, April 30 – May 4, 2006, Ft. Lauderdale, FL).

Uchino *et al.* designed a poly(vinyl alcohol) hydrogel crosslinked to an amniotic membrane (AM) to form a scaffold to support epithelial cell proliferation while providing a functional barrier similar to the basement membrane *in vivo* (48). This hydrogel was found to support the differentiation of epithelial cells; however, when transplanted into rabbit stroma, some eyes developed epithelial defects that resulted in the disintegration of AM and inflammation. Stabilizing the AM with alternative crosslinkers will be investigated in the future.

Some studies have implanted stromal equivalents to assess *in vivo* behavior. Hu *et al.* investigated the integration of poly-glycolic acid (PGA) scaffolds seeded with adherent corneal stromal cells into rabbit cornea stroma (49) to assess the potential reduction in tissue transparency due to the synthetic scaffold. Green fluorescent protein labeled stromal cells were harvested, mixed with PGA fibers, and implanted into the corneas of female rabbits. Abundant green fluorescent protein expression within the reconstructed corneas indicated that the engineered stromal tissue was formed by the implants and not the stromal cells local to the host cornea. The reconstructed cornea was observed to be nearly transparent to the naked eye after a period of 8 wk (Fig. 4). The main drawback to this technique was that PGA implantation caused the cornea to

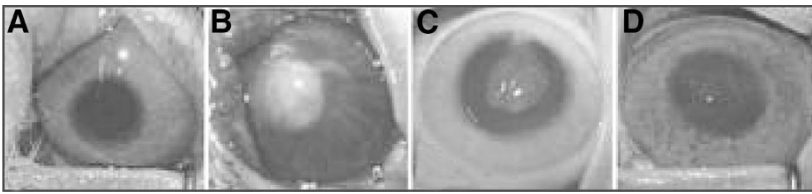


Figure 4. Sequence of photos indicating the increased transparency of the tissue engineered cornea over a period of eight weeks. (A) Before the operation, (B) immediately postoperation and (C) 4 wk, (D) 8 wk after operation. Image reprinted from Hu *et al.*, Tissue Eng 11:1710–1717, Copyright © 2005 Mary Ann Liebert, Inc., with permission.

thicken. Although this technique might prove suitable for rabbit corneas, the feasibility in a clinical setting is yet to be determined.

ADVANCES IN TISSUE-ENGINEERED CORNEAL EQUIVALENTS

In 1999, Griffith *et al.* published a landmark paper on corneal tissue engineering in *Science* (50). In this publication, she demonstrated the development of a collagen-chondroitin sulfate-based hydrogel, which was seeded with immortalized human corneal stromal keratocytes and covered on top and bottom with layers of immortalized human corneal epithelial and endothelial cells, respectively; these constructs were then cultured in standard tissue culture mediums with the addition of protease inhibitors and ascorbic acid and epithelial cells were exposed to an air-liquid interface after reaching confluence. Key to this approach was the electrophysiological pre-screening of the cell lines for whole-cell currents similar to those found in normal cells. In her *in vitro* evaluation of the constructs, Griffith showed that the constructs resembled the gross morphology (including a stratified epithelium) and transparency of a normal human cornea (Fig. 5). This effort has since led to an entire body of work on corneal tissue engineering at the University of Ottawa by Griffith and collaborators, which has continued to break new ground. Further

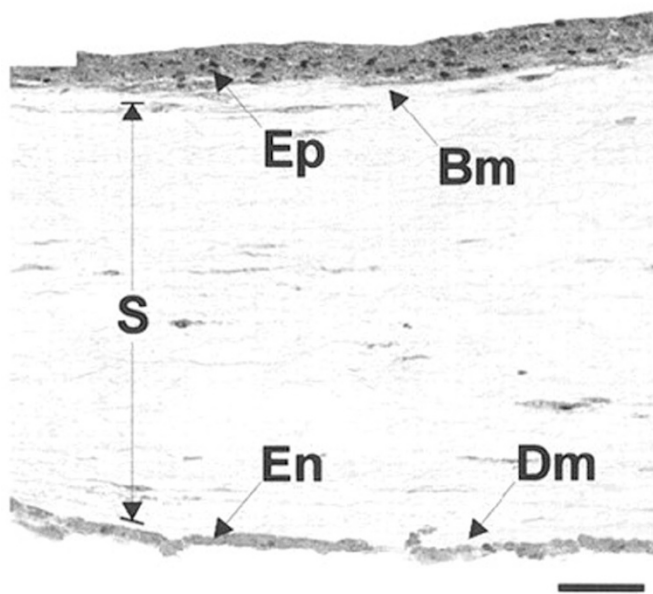


Figure 5. A tissue-engineered cornea which closely resembles the native cornea, with three distinct layers: epithelium (Ep), stroma (S), endothelium (En), separated by Bowman's (Bm) and Descemet's (Dm) membranes. Adapted from Griffith *et al.*, Science 286:2169–2172, Copyright © 1999 The American Association for the Advancement of Science, with permission.

studies on this model indicated that 0.02% glutaraldehyde crosslinking increased the tensile strength of the matrix (from 800 to 1900 kPa), the inclusion of 20% type C chondroitin sulfate increased transparency (from 55 to 66%, with maximal effect seen with addition of 1.2% CS-C), and the addition of ascorbic acid to the growth medium significantly increased collagen synthesis in crosslinked matrices (34).

A major focus within Griffith's work is the innervation of tissue-engineered corneas, as innervation has been shown to be integral to epithelial cell health and function, as well as corneal wound healing. The model used for this work included mouse embryonic dorsal root ganglia (DRG) seeded in a collagen-CS ring surrounding a stromal equivalent and epithelial layer from the original model, cultured for 10 days (51). The optimized culture system contained 100 ng/mL nerve growth factor, as well as a gradient of laminin from bottom to top of 0–20 $\mu\text{g/mL}$, resulting in guided neurite outgrowth from the DRGs through the stromal equivalent and into the epithelial layer. The nerve morphology was consistent with normal corneas, with clear bifurcations, parallel nerve bundles, and both beaded and smooth nerve fingers; nerves appeared to invaginate individual epithelial cells (Fig. 6). Additionally, neural functionality was demonstrated by the presence of active sodium channels, the generation of action potentials upon stimulation of DRGs, as well as significant response to neurotoxin. Innervation also stimulated twofold epithelial and keratocyte proliferation, better epithelial stratification, the establishment of a protective mucin layer by day 10, and a greater resistance to toxic agents, when compared with noninnervated constructs (52). In a further effort to improve corneal innervation and epithelial reconstruction, the group developed a copolymer [poly(*N*-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide), PNiPAAm-coAAc-coASI, designated TERP and an analog containing the YIGSR cell adhesion motif, designated TERP-5 (53,54). These materials were reacted with collagen to form crosslinked gels, which were then cast on top of the standard collagen-CS matrices; this construct was then surrounded by a collagen-CS ring, which was seeded with DRGs. In *in vitro* evaluations with immortalized epithelial cells, the TERP-5 constructs provided far superior optical clarity with direct transmission (>80%) and backscatter properties (<10%) superior to collagen-only constructs and similar to normal human corneas. Additionally, a twofold increase in epithelial stratification and a threefold increase in nerve density was observed. In a micropig lamellar keratoplasty model, TERP-5 constructs (without supporting or surrounding collagen-CS elements) showed complete re-epithelialization within 1 wk, new nerve ingrowth, and epithelial and anterior stromal morphologies (including a regenerated basement membrane) which were comparable to

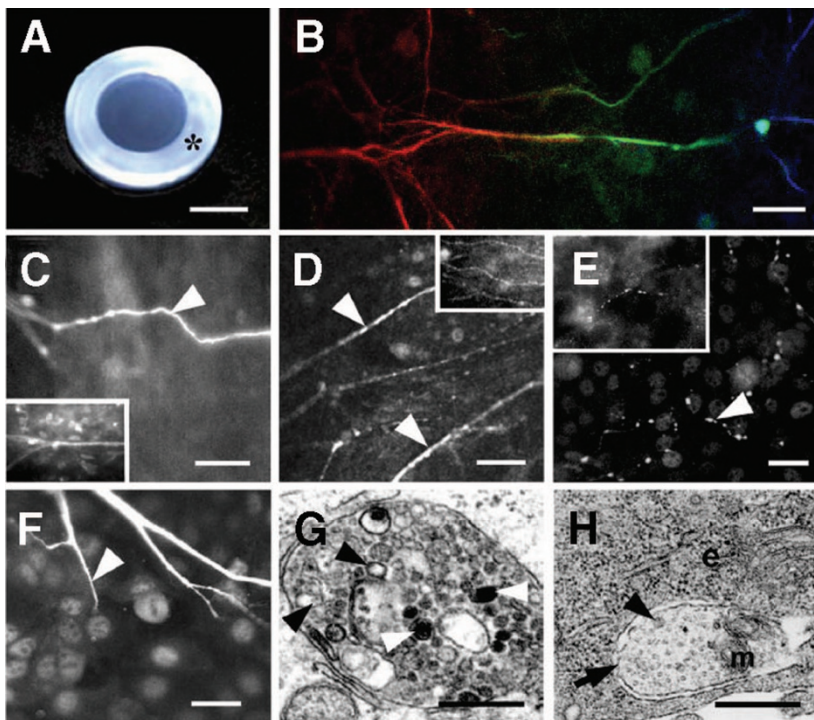


Figure 6. From the gross model to cellular invagination: images of tissue engineered (TE) corneal innervation. (A) Gross appearance of central, transparent TE cornea surrounded by opaque collagen-CS ring seeded with DRGs. (B) Bifurcating nerve extending from DRG through TE stroma and to epithelialized surface; red, 60 μm ; green, 40 μm ; and blue, 20 μm from the surface. (C) Nerves in TE cornea grossly resemble those found in the normal human cornea (insets), and show (D) parallel fibers, (E) beaded fibers, (F) and smooth fibers. (G) Nerve fiber terminal within the TE epithelium (H) Arrow shows a nerve fiber entering an epithelial cell. Scale bars: (A) 1 cm; (B, C) 10 μm ; (D) 20 μm ; (E, F) 10 μm ; (G) 0.3 μm ; (H) 0.5 μm . Reprinted from Suuronen *et al.*, FASEB J 18:170–172, Copyright © 2004 The Federation of American Societies for Experimental Biology, with permission.

allografts; within 21 d, touch sensitivity had returned to preoperative levels, indicating the restoration of neural function. In an interesting additional application of the TERP material, TERP was blended with fibrinogen, and growth factors to support angiogenesis, and was seeded with isolated DRGs and human umbilical vein endothelial cells (HUVECs), to form a scleral model; this was molded into a ring and the center was filled with a keratocyte-seeded collagen-CS corneal equivalent, with epithelial cells seeded on top (55). *In vitro*, this appeared to be a good scleral model, as HUVECs led to angiogenesis within the sclera, but the vasculature did not penetrate into the corneal regions; however, nerves originating from the DRGs extended through the sclera and into the corneal stroma and epithelial layers with normal morphologies.

Recently, Griffith *et al.* have developed a simple carbodiimide (EDC/NHS) crosslinked collagen based hydrogel, which is optically clear (99% light transmission), and has a refractive index and glucose permeability comparable to the normal human cornea (56,57). In *in vitro* evaluations, no local cytotoxicity, genotoxicity, or systemic cytotoxicity was observed, and epithelial growth rates were unaffected. In an *in vivo* rabbit model, re-epithelialization occurred in 1 week, with only 1 of 24 transplants showing a slight corneal haze 6 mo after surgery; by 3 mo, stromal cells were present in the construct, and the morphology and cellularity appeared normal by 6 mo. In a pig study, innervation was present by 3 mo, and a subepithelial nerve plexus was visible by 6 mo; an additional *in vivo* confocal microscopy study using these constructs defined a pattern of innervation beginning by 2 months in the deep anterior stroma and progressing to the recovery of preoperative nerve density in the subbasal layer by 1 year after transplantation (58). This construct's ability to

serve as a full thickness corneal graft was evaluated in a murine model with full penetrating keratoplasty (59). Although the grafts were mechanically stable, the recipient eyes remained transparent for only 10 days, and then became progressively opaque; a significant inflammatory response was observed, resulting in the formation of a retro-corneal fibrin membrane below the graft with infiltrating myeloid cells. Treatment with sodium citrate initially retarded the fibrin sheath formation, but lost its effect by day 6. Two months posttransplant, significant levels of anti-porcine type 1 collagen antibodies were detected, indicating a continued immune response.

This set of publications demonstrates the promise within the field of corneal tissue engineering; the ability to develop stable, transparent constructs that successfully epithelialize, innervate, and integrate with host tissue, is critical to developing a true, functional tissue-engineered corneal replacement. Major issues emerging within the field include the incorporation of endothelial cells into these constructs and control of the immune response. While much work has been done on isolated endothelial cells, it will be important to ensure that they may be integrated into the constructs in a manner that retains their functional properties, and their ability to protect the stroma and epithelium from some portion of the inflammatory response.

CONCLUSIONS AND FUTURE CHALLENGES

Much progress has been made in the field of corneal tissue engineering in recent years. We have been successful in harnessing the power of native cells to produce a stroma-like ECM, as well as to produce epithelial and endothelial cell

sheets complete with intact basement membrane. We have been able to create collagen-based engineered matrices, which support cell growth and exhibit appropriate optical properties. In addition, many strides have been made in improving the mechanical properties of both collagen and noncollagen matrices. Recent interest in growing constructs under stress shows promise for recreating the intricate structure of the cornea as well as influencing cell behavior. One group has made important contributions in terms of the innervation of a corneal equivalent. In the future, we will need to integrate these approaches to create a construct of appropriate thickness and mechanical strength. Further studies are required on the cellular response to these matrices to determine whether the wound healing phenotype is resolved over time in culture. Lastly, studies on reducing the inflammatory response to implanted materials will be crucial to the clinical success of any cornea replacement.

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