Published in final edited form as:

Biomaterials. 2012 February ; 33(5): 1343–1352. doi:10.1016/j.biomaterials.2011.10.055.

The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells

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Abstract

Corneal stroma is an avascular connective tissue characterized by layers of highly organized parallel collagen fibrils, mono-disperse in diameter with uniform local interfibrillar spacing. Reproducing this level of structure on a nano- and micro-scale may be essential to engineer corneal tissue with strength and transparency similar to that of native cornea. A substrate of aligned poly(ester urethane) urea (PEUU) fibers, 165±55 nm in diameter, induced alignment of cultured human corneal stromal stem cells (hCSSC) which elaborated a dense collagenous matrix, 8–10 µm in thickness, deposited on the PEUU substratum. This matrix contained collagen fibrils with uniform diameter and regular interfibrillar spacing, exhibiting global parallel alignment similar to that of native stroma. The cells expressed high levels of gene products unique to keratocytes. hCSSC cultured on PEUU fibers of random orientation or on a cast film of PEUU also differentiated to keratocytes and produced abundant matrix, but lacked matrix organization. These results demonstrate the importance of topographic cues in instructing organization of the transparent connective tissue of the corneal stroma by differentiated keratocytes. This important information will help with design of biomaterials for a bottom-up strategy to bioengineer spatially complex, collagen-based nano-structured constructs for corneal repair and regeneration.

Keywords

stem cell; collagen; nano-structured; topographic cues; electrospinning

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1. Introduction

Trauma, bacterial and viral infections, and heritable conditions lead to loss of corneal function and visual impairment in over ten million individuals world-wide [1]. Tissue accessibility and immune privilege of the cornea have led to the widespread practice of grafting allogenic corneal tissue to replace scarred or damaged corneas. The short-term success of this procedure is high, making the cornea the most frequently transplanted organ. In spite of this success, ocular inflammation prevents grafting in many eyes. Even with successful grafts, long-term (i.e. 10 year) graft survival may be as low as 64% [2]. Once a cornea is rejected, acceptance of a subsequent graft is often unsuccessful. Additionally, the distribution and supply of donor tissues are limited or non-existent in many parts of the world.

Limitations in the use of fresh corneal tissue for transplantation have led to a focus on other means of replacing pathologic corneal tissue. Approaches have included development of fully artificial keratoprotheses, use of decellularized tissue scaffolding from animal or human sources, and use of non-cellularized crosslinked collagen as corneal replacements [3,4]. Although some of these approaches are in currently practice, none has had the broad success and acceptance of fresh tissue transplantation. One approach not yet realized is that of a fully bioengineered cornea. Such an organ would be composed of the three characteristic tissues of the cornea, epithelium, endothelium and stroma, maintained and assembled in vitro. A proof of concept for this approach was demonstrated by Griffith et al in 1999 [5], but progress toward a working model has not been rapid. The difficulty in producing such a construct has been largely that of generating corneal stroma in vitro with strength and transparency equivalent to that of native tissue.

Corneal stroma is a dense connective tissue comprising 90% of the cornea. The stroma consists of multiple collagenous lamellae interspersed with a population of specialized mesenchymal cells, the keratocytes. Within each lamella collagen fibrils are parallel, tightly packed and highly uniform in diameter. Collagen fibril diameter and interfibrillar spacing are mediated by a specialized class of corneal proteoglycans. Transparency of this tissue to light is attributed to the unique tight packing and uniform diameter of the collagen fibrils. Genetic diseases causing disruption of this arrangement, such as mucopolysaccharioses, result in severely opaque corneas early in life [6]. Uninterrupted collagen fibrils run parallel to the surface of the cornea and orientation of fibril direction varies in adjacent lamellae, thus providing very high isotropic lateral strength to the tissue. These unique physical properties of strength and transparency, essential for corneal function, rely both on the molecular composition of the tissue and its elegant ultrastructural organization.

Keratocytes, resident cells of the corneal stroma, are responsible for secreting a spectrum of unique matrix molecules [7] and for maintenance of the unique transparent stromal tissue. In vitro expansion of keratocytes inevitably results in loss of the unique phenotype of these cells and differentiation into fibroblasts, leading to secretion of a disorganized ECM typical to that found in corneal scars [8–10]. The inability to find cultured cells that produce the unique molecular components of corneal ECM in vitro has led to difficulty in generating a bioengineered stromal construct suitable for bioengineering corneas.

Over the past several years, Du and Funderburgh *et al* [11–13] characterized a population of stem cells from adult human corneal stroma (hCSCs) which maintains the ability to become keratocytes through a large number of population doublings [11]. Unlike corneal fibroblasts, hCSC can produce abundant ECM containing keratan sulfate and keratocan, unique components of cornea stroma [12]. In pellet cultures, these cells produce localized regions of stroma-like ECM but do not organize these components globally to produce

functional stromal tissue. Pursuing the general hypothesis that stem or progenitor cells capable of adopting a keratocyte phenotype could recapitulate the developmental process and generate stromal tissue in vitro, we specifically hypothesized that spatial cues from an aligned fibrous substrate, mimicking the native stromal ultrastructure, could guide hCSCs to synthesize and organize ECM consistent with human corneal stromal tissue. A randomly oriented fibrous substrate and cast film were used as controls to investigate the influence of surface topology. The polymeric substrate utilized was a biodegradable and non-cytotoxic poly(ester urethane)urea (PEUU) processed using electrospinning and solvent casting [14,15].

2. Materials and methods

2.1 Materials

The poly(ester urethane) urea (PEUU) was synthesized as described before [14]. The brief synthesis protocol is as following. Firstly, 1,4-diisocyanatobutane and polycaprolactone diol (PCL, $M_w=2000$) were reacted in dimethyl sulfoxide (DMSO, anhydrous grade) for 3 hr at 75 °C with the aid of tin 2-ethylhexanoate under the protection of an argon purge. After cooling to room temperature, the oligomer solution was drop-wise added by 1,4-diaminobutane under vigorous stirring. After 18 hr reaction at room temperature, the polymer solution was precipitated in distilled water. Then the precipitant was soaked in anhydrous 2-propanol for another 48 hr to remove DMSO and unreacted monomers. The yielded polymer was incubated in anhydrous ethanol for another 24 hr to remove water, and then further dried under vacuum at 40°C for one week in order to remove residual water. The yielded product was a white elastomer.

Human corneas unsuitable for transplant were obtained from the Center for Organ Recovery & Education (Pittsburgh, PA). Two corneas from a 51-year old donor were used in the experiments. Human corneal stromal stem cells were isolated from collagenase-digested limbal stromal tissue and expanded from clones in stem cell growth medium (SCGM) containing DMEM/MCDB-201 with 2% fetal bovine serum, 10 ng/mL epidermal growth factor, 10 ng/mL platelet-derived growth factor (PDGF-BB), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid (ITS), 1×10^{-8} M linoleic acid-bovine serum albumin, 0.1 mM ascorbic acid-2-phosphate, 10^{-8} M dexamethasone, 100 IU/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, 100 ng/ml cholera toxin. The characteristics of the cloned stem cells are similar to those of the side-population cells described previously [11] in terms of clonogenicity, keratocytic differentiation, multipotency and stem cell marker expression. Cells at passage six were used for the experiments. The experiments were repeated three times.

Antibodies used for immunostaining included anti-keratocan polyclonal peptide antibody (Kera C, gift from Winston Kao, University of Cincinnati, Cincinnati OH) [12], monoclonal antibody BSK-1 to keratan sulfate [16] (gift from Bruce Caterson, Cardiff University, Cardiff, UK), anti-collagen I monoclonal (Sigma-Aldrich), anti-collagen V monoclonal (Chemicon), and anti-collagen VI monoclonal (Chemicon). Fluorescently tagged secondary antibodies, Alexa Fluor-488 anti-mouse IgG, Alexa Fluor-546 antirabbit IgG, as well as nuclear dye DAPI were purchased from Invitrogen.

2.2 Substrate Preparation

The oriented fibrillar substrates were prepared by the electrospinning process. PEUU was dissolved in hexafluoroisopropanol (HFIP) under mechanical stirring at room temperature. The obtained polymer solution was fed by syringe pump (Harvard Apparatus) into a steel capillary (I.D. = 0.43 mm) suspended over an aluminum wheel collector 2 cm wide and 20

cm in diameter. A combination of two high-voltage generators (Gamma High Voltage Research) was employed with a high positive voltage (+10 kV) to charge the steel capillary containing the polymer solution, and a high negative voltage (−5 kV) to charge the aluminum wheel collector. The distance between the tip of the steel capillary and the top of the aluminum wheel collector was 15 cm. The volume flow rate was mL/hr. A 5.0 wt% PEUU solution was electrospun with wheel rotational speeds of 2000 and 150 rpm in order to induce fiber orientation or not, respectively. The fibrillar substrates utilized were 200 μm thick.

Cast PEUU films were prepared as a control substrate with 5.0 wt% PEUU in HFIP poured into a Teflon™ casting dish, where the solvent evaporated to yield semitransparent, mechanically robust films with a prescribed thickness of 200 μm. All of the substrates were dried in a vacuum oven at room temperature for one week to eliminate the solvent completely.

2.3 Cell culture

The scaffold was punched into 25 mm diameter discs which were placed in wells of 24-well culture plates. The discs were sterilized by ultraviolet radiation (254 nm) in a cell culture hood for 20 minutes on each side. The hCSCs were statically seeded on the substrates at a density of 7.0×10^4 cells/cm² and incubated in SCGM.

After three days incubation in SCGM, hCSCs were transferred into keratocyte differentiation medium consisting of Advanced DMEM (Invitrogen) supplemented with 10 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 0.1 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich), L-glutamine (1×GlutaMax™-1; Invitrogen), 50 μg/mL gentamicin (Invitrogen), 100 μg/mL penicillin (Mediatech), which was changed twice per week for up to 6 weeks.

2.4 Two-photon Fluorescent Microscopy

Multiphoton microscopy was performed using an Olympus FV 1000 multiphoton microscope (Center Valley PA). This is an upright fixed stage microscope equipped with a large area motorized stage. Specimens were mounted in a home built imaging chamber, and 3 dimensional image sets collected with a 25X 1.0 NA objective specifically designed for multiphoton microscopy. The microscope laser was tuned to 830 nm which is ideal for collecting the second harmonic generation signal and the Cytex Green signal which is used to label the nuclei within cells. The images are collected as 3 dimensional data sets (section spacing 1 μm), the number of sections in each stack vary from specimen to specimen but include the full thickness of the specimen (between 8 and 14 μm). Image stacks once collected are processed into 3D stacks using Imaris (Bitplane).

2.5 Electron Microscopy

Cellular and extracellular matrix morphology as well as that of the underlying substrates was imaged with scanning electron microscopy (SEM). Specimens were fixed in cold 2.5% glutaraldehyde (EM grade, Taab Chemical) in 0.1 M PBS pH 7.3. The specimens were rinsed in PBS, post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) with 0.1% potassium ferricyanide (Fisher), dehydrated through a graded series of ethanol washes followed by a hexamethyldisilazane wash. The yielded sample was imaged at 5 kV by a Jeol JSM-6330F Scanning Electron Microscope.

Transmission Electron Microscopy (TEM) was used to image the cross-sectional microstructures of the deposited ECM. The specimens were fixed and dehydrated as for SEM with the exception that instead of hexamethyldisilazane treatment, samples were

embedded in Epon (made from dodecenyl succinic anhydride, nadic methyl anhydride, Scipoxy 812 Resin and 2,4,6-tris(dimethylaminomethyl)phenol, Energy Beam Sciences) and ultrathin sections (65 nm) were cut parallel and perpendicular to the alignment of the underlying fibrillar substrates. These were stained with 2% uranyl acetate (Electron Microscopy Sciences, and methanol, Thermo-Fisher Scientific) and 1% phosphotungstic acid (Sigma-Aldrich), pH 3.2. The sections were examined and photographed at 80kV on a Jeol 1011 transmission electron microscope.

2.6 Gene Expression

RNA of the differentiated hCSCs was isolated using the RNeasy mini kit (Qiagen). RNA was treated with DNase I (Ambion) and was concentrated by alcohol precipitation. RNA (400 ng) was transcribed to cDNA in a 50 μ L reaction containing 1 $\times$ PCR II buffer (Roche Applied Science), 5 mM $MgCl_2$, 200 μ M dNTP mixture (Roche), 2.5 μ M random hexamers (Invitrogen), 0.4 U RNase inhibitor, and 60 U SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR of cDNA was performed with direct dye binding (SYBR Green; Applied Biosystems) according to the manufacturer's instruction. Reactions were carried out on triplicate samples for 40 cycles of 15 seconds at 95 $^{\circ}$ C and 1 minute at 60 $^{\circ}$ C after initial incubation at 95 $^{\circ}$ C for 10 minutes. Reaction volume was 20 μ L. The reactions contained 1 $\times$ PCR buffer (Applied Biosystems), 3 mM Mg^{2+} , 200 μ M dATP, dCTP, dGTP, and 400 μ M dUTP, 0.025 U/mL AmpliTaq Gold polymerase, 1.6 μ L cDNA and forward and reverse primers at optimized concentrations. Amplification of 18S rRNA was carried out for each cDNA as a qualitative external control. A dissociation curve for each SYBR-based reaction was generated on a real-time thermocycler (Gene-Amp ABI Prism 7700 Sequence Detection System; Applied Biosystems) as a means to confirm that there was no nonspecific amplification. Amplification of 18S rRNA was performed for each cDNA (in triplicate) for normalization of RNA content. Relative mRNA abundance was calculated as the C_t for amplification of a gene-specific cDNA minus the average C_t for 18S expressed as a power of 2 ($2^{-\Delta C_t}$). Three independent gene-specific values thus calculated were averaged to obtain a mean $\pm$ SD. We chose ABCG2 as a genetic marker of corneal stromal stem cells, and keratocan, aldehyde dehydrogenase 3A1 (ALDH), prostaglandin D2 synthase (PTGDS), corneal N-acetylglucosamine-6-O-sulfotransferase (CHST6) and pyruvate dehydrogenase kinase isozyme 4 (PDK4) as genetic markers of keratocytes [11].

2.7 Immunostaining

The PEUU electrospun substrates seeded with hCSCs were fixed in 4.0 % paraformaldehyde in PBS at room temperature for 20 minutes, rinsed in PBS, and stored at 4 $^{\circ}$ C in PBS until further processing. Except for the case of keratocan staining, the fixed samples were incubated in 10% heat-inactivated goat serum at room temperature for one hour to block nonspecific binding, rinsed in PBS, and incubated with mouse-monoclonal primary antibodies diluted with 1% BSA in PBS overnight at 4 $^{\circ}$ C in a sealed moist box. For immunostaining of keratocan, the sample was first digested and blocked with keratanase (0.5 U/mL) (Sigma Aldrich) in 1% BSA in PBS for two hr at 37 $^{\circ}$ C, rinsed in PBS, and then incubated with primary goat-anti-human keratocan antibody overnight at 4 $^{\circ}$ C.

After three washes with PBS, secondary antibody Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 543-conjugated donkey anti-goat (1:2,500) (Invitrogen-Molecular Probes) together with 4',6-diamidino-2-phenylindole (DAPI) (0.5 ng/mL) (Roche Molecular Biochemicals) was added to the samples, and incubated for 2 hr at room temperature. The stained samples were placed in aqueous mounting medium (Thermo Fisher Scientific) and examined using an Olympus FluoView FV1000 confocal microscope.

2.8 Statistical Analysis

All of the results are expressed as mean $\pm$ standard deviation. One way ANOVA followed by post-hoc Newman-Keuls multiple comparison testing was utilized to evaluate the influence of substrate topography on gene expression of differentiated hCSCs, and $p < 0.05$ was considered significant.

3. Results

3.1 Cell Morphology on PEUU substrates varying with surface topography

Scanning electron micrographs (SEMs) of the generated PEUU substrates are seen in Figure 1(a, d and g) for aligned and random electrospun fibers and solvent cast film surfaces respectively. Fiber size was comparable for the two fibrous substrates (165 ± 55 nm for aligned, 171 ± 66 nm for random) whereas the cast film was comparatively flat and smooth with some local fluctuations induced by the processing, but no notable orientation. Cell viability and morphology after seeding were evaluated with Calcein AM staining. Fluorescent, viable hCSCs were highly elongated and uniaxially aligned on the oriented fibrous PEUU at one day of culture in SCGM (Figure 1b). After 3 days, hCSCs divided and proliferated to confluence while maintaining alignment (Figure 1c). For both the random fibrous (Figure 1e, 1f) and cast film surfaces (Figure 1h, 1i) hCSCs were less elongated and displayed no preferred orientation with ensuing culture.

3.2 Gene Expression of hCSCs after differentiation

After 3 days in SCGM, culture medium was switched to serum-free keratocyte differentiation medium. After 6 weeks culture in keratocyte differentiation medium, further characterization of the generated tissue construct was performed. First, the influence of the substrate on gene expression patterns of hCSC was evaluated as shown in Figure 2. Consistent with our previous report [12], hCSCs in keratocyte differentiation medium down-regulated the expression of ABCG2, a typical marker present in many adult stem cells, and substantially upregulated several markers of keratocytes, including keratocan (KERA), aldehyde dehydrogenase 3A1 (ALDH), prostaglandin D2 synthase (PTGDS), corneal N-acetylglucosamine-6-O-sulfotransferase (CHST6) and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4). These observations provide evidence that hCSCs were effectively differentiated into keratocytes in keratocyte differentiation medium. The adult stem cell phenotype of hCSCs was down-regulated to a greater extent on the aligned PEUU fibrous substrate than on the random fibrous substrate or cast film ($p < 0.05$). Differentiated hCSCs also showed differential expression of typical keratocyte markers based on the substrate, with stronger marker expression on aligned fibrous substrates or cast films versus random PEUU fibrous substrates. There was no significant difference in the expression of CHST6 and PTGDS between aligned fibrous scaffolds and cast film ($p > 0.05$).

3.3 Morphological assessment of extracellular matrix biosynthesized by differentiated hCSCs

3.3.1 Two-photon microscopy—Highly aligned molecules of Type-I collagen lead to strong birefringence [17,18]. In addition such aligned collagen features a second-order nonlinear susceptibility because of its structural high non-centrosymmetry, resulting in a strong second harmonic generation (SHG) [19–22]. Accordingly, the hCSC-secreted ECM when examined by two-photon microscopy (Figure 3) showed a very strong SHG signal (in red) when excited at $\lambda = 830$ nm. The ECM on the aligned substrate was organized into fibril bundles that were globally aligned across the substrate producing a strong SHG signal. In contrast, the fibril bundles on the random fibrous substrate and cast film were observed

generally to produce a weaker signal and to demonstrate a random alignment with only regional orientation.

3.3.2 Scanning electron microscopy—Detailed surface morphologies of hCSSCs and their elaborated ECMs from the micro- to nano-scale were observed with electron microscopy. On aligned fibrous PEUU, the hCSSCs were elongated, and uniformly oriented in the preferred fiber direction (Figure 4a). There were many dense, fine fibrils between cells on the scaffold aligned with high fidelity to hCSSC alignment. Figure 4b demonstrates the detailed microstructures of the hCSSC-secreted fibrillar ECM. The longitudinal axes of the fibrils were largely parallel to each other. Between the fibrils, there are numerous side branches that crosslink them into an integrated collagen construct. As shown in the insert of Figure 4b, a characteristic periodic banded structure was observed consistent with the *D*-band feature found in type-I collagen fibrils.

In Figure 4c a typical cell on the random fibrous substrate is seen with a dendritic cellular morphology common to the film, but not the aligned fibrous substrate. The secreted ECM on the random fibrous substrate was also less densely packed. As shown in Figure 4d, there was no preferred alignment in the lower magnification view. The distinct transverse banding pattern, characteristic of a Type-I collagen fibril, can also be seen along each fibril as shown in the insert of Figure 4d. The observations on the cast film were similar to those on the random fibrous substrate, as shown in Figure 4e and f. Individual fibrils secreted from hCSSCs on cast films did not appear to vary greatly from those on aligned and random fibrous sheets, and although some regional fibril alignment was observed, the degree and direction of this alignment was not consistent across microscopic fields observed.

3.3.3. Transmission electron microscopy—Since SEM visualization only allowed assessment of the top layer of hCSSC-secreted ECM organization, transmission electron microscopy was utilized to evaluate whether ECM distribution in the *z*-direction was consistent with physiological structures and varied between substrate types. Transmission electron micrographs of the hCSSC-secreted ECM in cross-section are shown in Figure 5. Due to the defined structural anisotropy, hCSSC-secreted ECM on aligned fibrous substrate was microtomed orthogonally along and across the fibril long axis. For samples cut across the fibril long axis, the hCSSC-secreted ECM was sandwiched by single cell layers and was ~8–10 μm thick, as shown in Figure 5a. No cells were found in this gap. When viewed at higher magnification (Figure 5b), all of the fibrils were found to be normal to the imaging plane. Within the ECM, dense fiber clusters were present with uniform fiber spacing, often as part of a pseudo-hexagonal lattice characteristic of native corneal stroma [23], (seen in the insert of Figure 5b). Fiber diameter (40.2 ± 2.7 nm) and spacing (60.9 ± 6.5 nm) within the clusters were analyzed by digital image processing with the distributions summarized in Figure 5d, 5e. For samples cut along the fiber long axis, fibrils were found to be parallel to the viewing plane and each other as shown in Figure 5c. Digital analysis summarized in Figure 5f revealed that the preferred angle (θ) was $89.8^\circ \pm 5.2^\circ$ demonstrating a highly orderly fibrillar organization. As shown in the insert of Figure 5c, uniform periodic banding could clearly be seen along each fiber with a periodicity of 67.6 ± 1.5 nm, which is very close to the characteristic *D*-spacing of native Type-I collagen. For hCSSCs cultured on random fibrous substrate and cast films a sandwiched ECM was also observed, as shown in Figure 5g and 5i respectively. However, in these cases the fibrils in the 4–6 μm thick ECM did not exhibit any preferred orientation. Under higher magnification (Figure 5h and 5j), it can be seen that fiber size was not uniform; however the characteristic axial *D*-periodicity was observed along the secreted fibers.

3.4 Analysis of extracellular matrix expression

Also examined was the expression of collagens and proteoglycans typifying the unique ECM of human corneal stromal tissue by whole mount immunohistochemical staining. In Figure 6a, 6a', 6a" it is seen that type-I collagen was abundant in the three secreted ECMs. Confirming the electron microscopy, the fibrous ECM deposited on the aligned fibrillar sheet exhibited high type-I collagen alignment. In addition, collagen V, collagen VI, keratan sulfate and keratocan were also detected as shown in Figure 6b–6e. All of these representative collagens and proteoglycans were also present in hCSSC-secreted ECM on random fibrous and cast film substrates, as shown in Figure 6a'–6e' and 6a"–6e". However, these fibrils on these two substrates demonstrated no preferred orientation. Structurally, the ECM on aligned fibrous substrates better approximated the flattened lamellae of corneal stromal tissue, e.g. one single layer construct with highly orderly uniform Type-I collagen fibrils. These results were in accordance with the observations from two-photon microscopy.

4. Discussion

Nano-structured collagen fibers represent the fundamental building blocks of many connective tissues. To engineer functional replacement tissues, recapitulating the process by which the underlying structural elements are ordered is highly desirable. Human corneal stroma possesses a unique organization of collagen fibrils with mono-disperse fiber diameter and uniform local interfibrillar spacing. Hierarchically, the stroma consists of ~200 acellular collagenous lamellae, each about 1.5~2.5 micrometer in thickness. The collagen fibrils of each lamella are parallel with one another, perpendicular to those of adjacent lamellae. As Ruberti *et al* pointed out [24], "the stroma is extremely organized on the nanoscale making it a very difficult and interesting basic materials engineering problem." In this study, we demonstrated hCSSC to have the potential to secrete ECM which recapitulates the orderly 3-D nano-structure of a single lamella.

As we previously reported [11,12], when hCSSC were transferred to serum-free medium with ascorbate, the cells expressed genes characteristic of differentiated keratocytes. Even though this occurred under all experimental conditions in this report, only the aligned fibrillar PEUU substrate effectively induced hCSSCs to elongate and align following the substrate fiber orientation as shown in Figure 1(b, c). In contrast, a random fibrous substrate and cast film surface did not induce a similar cellular alignment. This alignment of the cells was reflected in the alignment of collagen throughout the ECM layer. As this layer thickened, the cells lost contact with the substrate, suggesting that the topographic cue is required only to initiate the process of ECM organization. Once initiated, the cells appear able to maintain secretion of an organized ECM. Without the aligned substrate, these cells produced only small, localized regions of organized stroma-like ECM. The global organization induced by an aligned fibrillar substrate coupled with the fact that the substrate is not incorporated into the secreted ECM suggests that the substrate appears to be initiating (i.e., instructing) a natural process, not serving as a scaffold.

On the aligned fibrous substrate the ECM produced showed a high resemblance to native stroma. As shown in Figure 6, the ECM secreted by hCSSCs stained for Type-V collagen, Type-VI collagen, keratocan, and keratan sulfate, factors which are determinant in collagen fibril size and interfibrillar spacing. Type-V collagen is believed to hybridize with Type-I in human corneal stroma, with the Type-I/Type-V ratio determining the hybrid fibril diameter. In normal human corneal stroma this ratio is 4:1 [25,26], resulting in a 31 ± 0.8 nm diameter [23]. The observed larger fiber diameter in this study (40.2 ± 2.7 nm) could be due to a larger ratio of Type-I to Type-V collagen [25,27]. Keratocan is a cornea-specific member of the small leucine-rich proteoglycan family abundant in corneal stroma. Members of this family bind to collagen fibers, with highly charged glycosaminoglycan chains extending into the

interfibrillar space mediating the distance between adjacent collagen fibers. Possible evidence of such a phenomenon was observed in the electron micrographs of Figure 4. Imbalance of the corneal proteoglycans usually leads to the disorganized fibril spacing [28–33], and corneal fibroblasts produce a spectrum of proteoglycans lacking keratocan similar to that of corneal scar tissue [7–10,34]. The presence of keratan sulfate and keratocan, and the observed interfibrillar collagen spacing of 60.9 ± 6.5 nm, were very similar to that observed in human stroma.

Global organization of a collagenous matrix by corneal cells in vitro has not been previously reported. Passaged keratocytes (fibroblasts) produce large but less organized regions of aligned collagen fibers in a Transwell culture model [35] and also when embedded in magnetically aligned collagen [36]. Our use of cells that express keratocyte phenotype and secrete molecular components unique to corneal ECM was clearly an improvement over fibroblasts; however, the keratocyte phenotype alone does not seem to be sufficient to produce organized ECM. hCSSC differentiated in pellets [12] or on non-aligned PEUU (this study) did not produce globally aligned stromal tissue in spite of the observation that these cells are expressing a qualitatively similar pattern of genes as those on aligned matrix. The inevitable conclusion of these experiments is that keratocytes require topographic cues to produce the unique ECM characteristic of corneal stroma. This observation clearly provides an important principal for further bioengineering of this tissue.

Besides surface topography, other cues that could affect ECM elaboration are the stiffness and chemistry of the substrates. Koo *et al* [37] studied the response of human corneal fibroblasts to micro- and nano-gratings on chitosan and PDMS and reported that the proliferation and elongation of keratocytes to be almost independent of materials chemistry and substrate stiffness. Only grating width (i.e., topographical cues) were involved in determining keratocyte response. These findings do not eliminate the possibilities that materials can make a difference in stromal bioengineering. In this study, we utilized PEUU due to its handling properties, lack of cytotoxicity biodegradability and processability. Gill *et al* [38,39] and Lawrence *et al* [40] demonstrated that surface-patterned silk films can also effectively induce the elongation of corneal fibroblasts, and result in strong expression of corneal extracellular matrix including collagen-V, decorin and biglycan. Magnetically aligned collagen [36], compressed collagen gels [41], and chemically crosslinked collagen [4] have also been used in corneal applications. Determining the advantages of each of these materials may require comparisons using standardized cell type and culture conditions.

A marked difference between the constructs reported in this work and the native tissue is that while the thickness. (e.g. 8–10 μ m) is greater than an individual lamella (2 μ m), it is much less than that of native human corneal stromal tissue. Constructs generated from corneal fibroblasts seeded on a micro-patterned poly(styrene-*b*-butadiene *b*-styrene) substrate [42] as well as the Transwell models [35] are reported to produce multiple lamellae, but in the current case only one cell-ECM layer was produced during the six weeks of culture. This difference may be attributable to our use of serum-free culture medium, which is less mitogenic than serum-containing media. Since the native corneal stroma is comprised of multiple, orthogonal layers of collagen-rich ECM, the approach here is of value in that highly aligned, collagen rich individual layers can be constructed. These layers might subsequently be combined (stacked) in an orthogonal or offset fashion to achieve the desired construct thickness [39].

A final consideration of this experimental approach that may be of advantage to ultimate clinical application, is the immune suppression that appeared to be exhibited by hCSSC in earlier studies. Du *et al* [13] showed that when these cells were transferred to mouse corneal stroma they remained in place for over one year, whereas human corneal fibroblasts

underwent T-cell mediated rejection within 2 weeks [13]. Immune suppression by other types of adult stem cells has been reported as well. Even though allogeneic transplantation of cornea without anti-rejection drugs is a standard practice, loss of grafts by rejection ultimately mirrors that of other organs [43]. Producing a bioengineered tissue from cells with reduced recognition by the immune system may have distinct advantages for its application to the human population.

5. Conclusion

In summary, we successfully prepared a highly ordered collagen nano-construct by employing a substrate with fibrillar morphology on a sub-micron scale. This amenable microenvironment facilitated the secretion by human corneal stromal stem cells (hCSCs) of a type-I collagen-based ECM that exhibited many of the key structural characteristics of native human cornea stromal tissue. Substrate topography appeared to play a critical role in initiating this ECM expression since a material equivalent, non-aligned fibrillar substrate and smooth film did not recapitulate the overall structural and compositional morphology of the ECM. Importantly, it was not necessary for the substrate to maintain contact with the cells to achieve this effect. These results, therefore, provide insights into the role of the extracellular environment in instructing cellular behavior and ECM nano-assembly. The study also represents an important step in a bottom-up strategy to bioengineer spatially complex collagen-based nano-structured constructs for corneal repair and regeneration.

Acknowledgments

The authors are very grateful to the support from Center of Biologic Imaging (CBI) of University of Pittsburgh. The assistance of Mr. Gregory Gibson and Mrs. Ming Sun with two-photon microscopy and sample preparation for transmission electronic microscopy is gratefully acknowledged. This work was kindly supported by the Ocular Tissue Engineering and Regenerative Ophthalmology (OTERO) program of the UPMC Eye Center and the McGowan Institute for Regenerative Medicine, Research to Prevent Blindness Inc. and NIH grant EY016415 (to JLF).

References

- Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ.* 2001; 79:214–221. [PubMed: 11285665]
- Borderie VM, Boelle PY, Touzeau O, Allouch C, Boutboul S, Laroche L. Predicted long-term outcome of corneal transplantation. *Ophthalmology.* 2009; 116:2354–2360. [PubMed: 19815285]
- McLaughlin CR, Tsai RJ, Latorre MA, Griffith M. Bioengineered corneas for transplantation and in vitro toxicology. *Front Biosci.* 2009; 14:3326–3337. [PubMed: 19273277]
- Fagerholm P, Lagali NS, Merrett K, Jackson WB, Munger R, Liu Y, et al. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. *Sci Transl Med.* 2010; 2:46–61.
- Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, et al. Functional human corneal equivalents constructed from cell lines. *Science.* 1999; 286:2169–2172. [PubMed: 10591651]
- Giugliani R, Federhen A, Rojas MV, Vieira T, Artigalas O, Pinto LL, et al. Mucopolysaccharidosis I, II, and VI: brief review and guidelines for treatment. *Genet Mol Biol.* 2010; 33:589–604. [PubMed: 21637564]
- Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L, Roth MR. Proteoglycan expression during transforming growth factor beta-induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem.* 2001; 276:44173–44178. [PubMed: 11555658]
- Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea.* 1996; 15:505–516. [PubMed: 8862928]

9. Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci.* 1999; 40:1658–1663. [PubMed: 10393032]
10. Long CJ, Roth MR, Tasheva ES, Funderburgh M, Smit R, Conrad GW, et al. Fibroblast growth factor-2 promotes keratan sulfate proteoglycan expression by keratocytes in vitro. *J Biol Chem.* 2000; 275:13918–13923. [PubMed: 10788517]
11. Du Y, Funderburgh ML, Mann MM, SundarRaj N, Funderburgh JL. Multipotent stem cells in human corneal stroma. *Stem Cells.* 2005; 23:1266–1275. [PubMed: 16051989]
12. Du Y, Sundarraj N, Funderburgh ML, Harvey SA, Birk DE, Funderburgh JL. Secretion and organization of a cornea-like tissue in vitro by stem cells from human corneal stroma. *Invest Ophthalmol Vis Sci.* 2007; 48:5038–5045. [PubMed: 17962455]
13. Du Y, Carlson EC, Funderburgh ML, Birk DE, Pearlman E, Guo N, et al. Stem cell therapy restores transparency to defective murine corneas. *Stem Cells.* 2009; 27:1635–1642. [PubMed: 19544455]
14. Guan J, Sacks MS, Beckman EJ, Wagner WR. Synthesis, characterization, and cytocompatibility of elastomeric, biodegradable poly(ester-urethane)ureas based on poly(caprolactone) and putrescine. *J Biomed Mater Res.* 2002; 61:493–503. [PubMed: 12115475]
15. Stankus JJ, Guan J, Wagner WR. Fabrication of biodegradable elastomeric scaffolds with sub-micron morphologies. *J Biomed Mater Res A.* 2004; 70:603–614. [PubMed: 15307165]
16. Akhtar S, Kerr BC, Hayes AJ, Hughes CE, Meek KM, Caterson B. Immunochemical localization of keratan sulfate proteoglycans in cornea, sclera, and limbus using a keratanase-generated neoepitope monoclonal antibody. *Invest Ophthalmol Vis Sci.* 2008; 49:2424–2431. [PubMed: 18515583]
17. Naylor EJ. Polarized light studies of corneal structure. *Br J Ophthalmol.* 1953; 37:77–84. [PubMed: 13032358]
18. Wolman M, Kasten FH. Polarized light microscopy in the study of the molecular structure of collagen and reticulin. *Histochemistry.* 1986; 85:41–49. [PubMed: 3733471]
19. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol.* 2002; 2:872–880. [PubMed: 12415310]
20. Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol.* 2003; 21:1369–1377. [PubMed: 14595365]
21. Williams RM, Zipfel WR, Webb WW. Interpreting second-harmonic generation images of collagen I fibrils. *Biophys J.* 2005; 88:1377–1386. [PubMed: 15533922]
22. Yasui T, Tohno Y, Araki T. Characterization of collagen orientation in human dermis by two-dimensional second-harmonic-generation polarimetry. *J Biomed Opt.* 2004; 9:259–264. [PubMed: 15065889]
23. Meek KM, Leonard DW. Ultrastructure of the corneal stroma: a comparative study. *Biophys J.* 1993; 64:273–280. [PubMed: 8431547]
24. Ruberti JW, Zieske JD. Prelude to corneal tissue engineering - gaining control of collagen organization. *Prog Retin Eye Res.* 2008; 27:549–577. [PubMed: 18775789]
25. Birk DE, Fitch JM, Babiarz JP, Doane KJ, Linsenmayer TF. Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. *J Cell Sci.* 1990; 95 (Pt 4):649–657. [PubMed: 2384532]
26. Birk DE. Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. *Micron.* 2001; 32:223–237. [PubMed: 11006503]
27. Birk DE, Fitch JM, Linsenmayer TF. Organization of collagen types I and V in the embryonic chicken cornea. *Invest Ophthalmol Vis Sci.* 1986; 27:1470–1477. [PubMed: 3531080]
28. Funderburgh JL, Caterson B, Conrad GW. Keratan sulfate proteoglycan during embryonic development of the chicken cornea. *Dev Biol.* 1986; 116:267–277. [PubMed: 2942429]
29. Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Carroll H. Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. *J Cell Biol.* 1998; 141:1277–1286. [PubMed: 9606218]
30. Funderburgh JL. Keratan sulfate: structure, biosynthesis, and function. *Glycobiology.* 2000; 10:951–958. [PubMed: 11030741]

31. Kao WW, Liu CY. Roles of lumican and keratocan on corneal transparency. *Glycoconj J*. 2002; 19:275–285. [PubMed: 12975606]
32. Carlson EC, Liu CY, Chikama T, Hayashi Y, Kao CW, Birk DE, et al. Keratocan, a cornea-specific keratan sulfate proteoglycan, is regulated by lumican. *J Biol Chem*. 2005; 280:25541–25547. [PubMed: 15849191]
33. Chakravarti S, Zhang G, Chervoneva I, Roberts L, Birk DE. Collagen fibril assembly during postnatal development and dysfunctional regulation in the lumican-deficient murine cornea. *Dev Dyn*. 2006; 235:2493–2506. [PubMed: 16786597]
34. Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. *J Biol Chem*. 2003; 278:45629–45637. [PubMed: 12933807]
35. Guo X, Hutcheon AE, Melotti SA, Zieske JD, Trinkaus-Randall V, Ruberti JW. Morphologic characterization of organized extracellular matrix deposition by ascorbic acid-stimulated human corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2007; 48:4050–4060. [PubMed: 17724187]
36. Builles N, Janin-Manificat H, Malbouyres M, Justin V, Rovere MR, Pellegrini G, et al. Use of magnetically oriented orthogonal collagen scaffolds for hemi-corneal reconstruction and regeneration. *Biomaterials*. 2010; 31:8313–8322. [PubMed: 20708260]
37. Koo S, Ahn SJ, Zhang H, Wang JC, Yim EKF. Human corneal keratocyte response to micro- and nano-gratings on chitosan and PDMS. *Cell Mol Bioeng*. 2011; 4:399–410.
38. Gil ES, Park SH, Marchant J, Omenetto F, Kaplan DL. Response of human corneal fibroblasts on silk film surface patterns. *Macromol Biosci*. 2010; 10:664–673. [PubMed: 20301120]
39. Gil ES, Mandal BB, Park SH, Marchant JK, Omenetto FG, Kaplan DL. Helicoidal multi-lamellar features of RGD-functionalized silk biomaterials for corneal tissue engineering. *Biomaterials*. 2010; 31:8953–8963. [PubMed: 20801503]
40. Lawrence BD, Marchant JK, Pindrus MA, Omenetto FG, Kaplan DL. Silk film biomaterials for cornea tissue engineering. *Biomaterials*. 2009; 30:1299–1308. [PubMed: 19059642]
41. Mi S, Khutoryanskiy VV, Jones RR, Zhu X, Hamley IW, Connon CJ. Photochemical cross-linking of plastically compressed collagen gel produces an optimal scaffold for corneal tissue engineering. *J Biomed Mater Res A*. 2011; 99:1–8. [PubMed: 21732526]
42. Guillemette MD, Cui B, Roy E, Gauvin R, Giasson CJ, Esch MB, et al. Surface topography induces 3D self-orientation of cells and extracellular matrix resulting in improved tissue function. *Integr Biol (Camb)*. 2009; 1:196–204. [PubMed: 20023803]
43. Council on Scientific Affairs. Report of the organ transplant panel: corneal transplantation. *JAMA*. 1988; 259:719–722. [PubMed: 3275820]

Figure 1. SEMs of (a) aligned fibrous PEUU, (d) random fibrous PEUU, (g) PEUU cast film. Confocal laser-scanning micrographs of hCSCs on (b, c) aligned fibrous, (e, f) random fibrillar, and (g, h) film substrates at day 1 (b, e, h) and day 3 (c, f, i). Fluorescent labeling was with the cell viability marker Calcein AM.

Figure 2.

Changes in gene expression of hCSCCs seeded on aligned fibrous substrates (black), random fibrous substrates (red) and cast films (green). mRNA abundance was compared with hCSCCs cultured in SCGM. Ratios of abundance of each transcript between hCSCCs seeded on different substrates cultured in keratocyte differentiation medium and in SCGM are expressed on a linear scale. Since KERA has no expression in hCSCCs cultured in SCGM, it is expressed in an absolute manner. Error bars show SD of three independent samples. For each gene, expression levels were significantly different between the studied substrates ($p < 0.05$), except for CHST6 and PTGDS on aligned fibrous substrate and cast film.

Figure 3.

Two-photon images of hCSSC-secreted ECM varying with: (a) aligned fibrous, (b) random fibrous, and (c) cast film PEUU. The second harmonic generation signal for collagens when excited at $\lambda=830\text{nm}$ is red. Nuclei are stained green.

Figure 4. SEMs of hCSCs and the elaborated ECM on the (a,b) aligned fibrous, (c,d) random fibrous, and (e,f) cast film substrates. The morphologies of hCSCs and secreted ECM are seen at the micron- to nano-scale. The banded structures along the fibrils are seen in the higher magnification insets of (b), (d) and (f) where the scale bar = 100 nm.

Figure 5.

Transmission electron micrographs of hCSSCs seeded on aligned fibrillar (a–c) random fibrillar (g–h), and cast (i–j) PEUU substrates for six weeks. TEM micrographs ((a) and (b)) revealed fibers from cells on the aligned scaffold exhibited high fidelity to a preferred orientation and long-range order. The fibers were normal to the viewing plane when the section crossed the cell long axis (a and b), whereas when the section was along the cell long axis, fibers were nearly parallel to each other (c). Digital analysis of the fiber diameter, fiber spacing and fiber orientation are seen in (d), (e) and (f), respectively. Inset in (c) shows determination of fibril orientation compared to a self-defined preferred angle. Fibers

generated on the random fibrillar substrate (g and h) and on the cast film (i and j) randomly distributed in the construct. The scale bar in the insert is 100 nm.

Figure 6.

Immunofluorescent micrographs of hCSSC-secreted ECM on (a–e) aligned fibrillar, (a'–e') random fibrillar and (a''–e'') cast film PEUU substrates after six week culture: (a, a', a'') collagen I, (b, b', b'') collagen V, (c, c', c'') collagen VI, (d, d', d'') keratocan and (e, e', e'') keratan sulfate. Nuclei were stained with DAPI (blue).