Published in final edited form as:

Exp Eye Res. 2014 March ; 120: 71–81. doi:10.1016/j.exer.2014.01.005.

Corneal Stromal Stem Cells versus Corneal Fibroblasts in Generating Structurally Appropriate Corneal Stromal Tissue

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Abstract

Recapitulation of human corneal stromal tissue is believed to be among the most challenging steps in engineering human corneal tissue because of the difficulty in reproducing its highly-ordered hierarchical ultrastructure, which imparts its robust biomechanical properties and optical transparency. In this study, we compared the feasibility of utilizing human corneal stromal stem cells (hCSCs) and human corneal fibroblasts (hCFs) in the generation of human corneal stromal tissue on a highly-aligned fibrous substrate made from poly(ester urethane) urea. In the serum-free keratocyte differentiation medium supplemented with FGF-2 (10 ng/mL) and TGF- β 3 (0.1 ng/mL), hCSCs successfully differentiated into keratocytes and secreted multilayered lamellae with orthogonally-oriented collagen fibrils, in a pattern mimicking human corneal stromal tissue. The constructs were 60–70 μ m thick and abundant in cornea-specific extracellular matrix (ECM) components, including keratan sulfate, lumican, and keratocan. Under the identical conditions, hCFs tended to differentiate into myofibroblasts and deposited a less-organized collagen-fibrillar construct in a pattern with similarities to corneal scar tissue due to a lack of cornea-specific ECM components. These observations demonstrated that hCSCs showed a much greater potential, under proper substrate and growth factor guidance, to facilitate the generation of a biological human cornea equivalent. Unlike hCSCs, hCFs were less responsive to these environmental cues and under identical culture conditions generated an ECM that poorly mimicked the native, functional tissue structure and composition.

Keywords

Stem Cells; Corneal Fibroblasts; Corneal Stroma; Ultrastructure; Tissue Engineering

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1. Introduction

Once corneal tissue is damaged or diseased, allograft cornea transplantation is the prevailing option to correct visual impairment. In the USA, there are more than 30,000 to 40,000 corneal transplantation operations performed every year. Although the short-term success of this procedure is high, long-term (i.e. 10 year) graft survival may be as low as 64%. (Borderie et al. 2009) Once a transplanted cornea is rejected, a subsequent graft is usually unacceptable. Furthermore, the use of LASIK surgery for refractive correction is reducing the future allogenic cornea donor supply. In many parts of the world, specifically developing countries, donor tissues are limited. Thus efforts to develop biological human corneal equivalents have been pursued by employing tissue engineering principles. (Germain et al. 2000; Ruberti and Zieske 2008; Shah et al. 2008)

So far, there have not been clinically viable full-thickness human corneal equivalents produced by tissue engineering methods, although some partial thickness lamellar keratoplasties have been attempted in animal models, (Li et al. 2003) and even in human clinical trials. (Fagerholm et al. 2010) A major challenge in successfully bioengineering cornea is the difficulty in reproducing the unique structure and composition of the stromal tissue. Occupying 90% of the corneal thickness, the stroma is comprised of 300~500 orthogonally oriented, highly aligned lamellae, which are formed from collagen fibrils of uniform size and regular inter-fibril spacing. This complex hierarchical ultrastructure is principally responsible for optical transparency and biomechanical properties of human cornea. (Maurice 1957; Benedek 1971; Ruberti et al. 2011)

Keratocytes are native resident cells of the corneal stroma, principally responsible for the maintenance of the unique transparent stromal tissue by secreting a spectrum of unique matrix molecules. However, these cells inevitably differentiate into corneal fibroblasts during expansion *in vitro* under serum-containing culture medium. Corneal fibroblasts lose the unique phenotype of keratocytes and secrete a disorganized extracellular matrix (ECM) typically found in corneal scars (Jester et al. 1996; Beales et al. 1999; Long et al. 2000). Fortunately, the discovery and isolation of human corneal stromal stem cells (hCSCs) (Du et al. 2005; Du et al. 2007; Du et al. 2009; Pinnamaneni and Funderburgh 2012) have made it possible to recapitulate the developmental process and generate stromal tissue *in vitro*. Unlike keratocytes, hCSCs do not down-regulate the typical mRNA expression patterns of stem and neural crest embryonic cells, nor do they lose the ability to adopt a keratocyte phenotype after a larger number of population doublings *in vitro*. (Du et al. 2005) When cultured in serum-free keratocyte differentiation medium (KDM), hCSCs differentiate into keratocytes and produce abundant cornea-specific ECM. In previous studies, it has been demonstrated that appropriate growth factor supplementation (i.e. FGF-2 and TGF- β 3) and the utilization of a highly aligned fibrous substrate are critical to engineering a well-organized, collagen fibril-based cornea-specific ECM. (Wu et al. 2012; Wu et al. 2013)

Corneal fibroblasts (i.e. stromal cells expanded in serum-containing culture media) lose characteristics of keratocytes and were long thought to lack the potential to regain keratocyte phenotype. (Pinnamaneni and Funderburgh 2012) More recent studies with human primary corneal fibroblasts (hCFs, unselected cells from the corneal stroma) have reported these cells capable of secretion and organization of a stroma-like ECM when cultured in 10% serum on a Transwell™ membrane system. Ascorbate and TGF- β 3 enhanced this secretion (Guo et al. 2007; Ren et al. 2008; Karamichos et al. 2010; Karamichos et al. 2011; Karamichos et al. 2012). Our previous reports were carried out using only adult stem cells from the corneal stroma. The object of our current study was to carry out a direct comparison of hCSCs versus hCFs to assess their ability in the generation of human corneal stromal tissue under identical experimental conditions, and to investigate whether the serum-free biomimetic

approach could guide hCFs to generate a cornea-specific ECM in the same manner as hCSSCs.

2. Material and Methods

2.1 Material

Poly (ester urethane) urea (PEUU), was prepared by two-step condensation reactions as previously reported.(Wu et al. 2012; Wu et al. 2013) The synthesized PEUU was dissolved in hexafluoroisopropanol (HFIP, Oakwood Product Inc) to prepare a 5 wt-% polymer solution that was then processed using electrospinning technique to obtain a highly aligned PEUU fibrous scaffold on a rapidly rotating mandrel using methods described previously. (Wu et al. 2012; Wu et al. 2013)

2.2 Culture of human corneal stromal stem cells and corneal fibroblast

Human corneal stromal stem cells (hCSSCs) were isolated from collagenase-digested limbal stromal tissue of human corneas unsuitable for transplant obtained from the Center for Organ Recovery & Education (Pittsburgh, PA). (Du et al. 2005) In brief, limbal stromal cells solubilized using collagenase digestion were initially cultured at clonal density (1×10^4 cells/cm²) in stem cell growth medium supplemented with 100 ng/ml cholera toxin (Sigma Chemicals, St. Louis, MO). Colonies of small polygonal cells were selected for further expansion. Clonal density of each passage was 1×10^4 cells/cm². As previously described, cells at passage six were used for the experiments.(Wu et al. 2012) To prepare human corneal fibroblasts (hCFs), collagenase-isolated human corneal stromal keratocytes were expanded in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and passaged 1:4 by trypsinization. hCFs at passage six were used for the experiments.(Garagorri et al. 2008; Du et al. 2009)

Discs of sterile PEUU fibrous material (25 mm diameter, 0.2 mm thick) were cut and anchored in wells of 24-well culture plates using silicone O-rings. hCSSCs and hCFs were seeded on the substratum at 5.0×10^4 cells/cm². hCSSCs were incubated with 1.0 mL of stem cell growth medium until confluent (usually 3 days).(Wu et al. 2012) hCFs were incubated in DMEM/F-12 containing 10 % FBS until confluent. After achieving confluence, both hCSSCs and hCFs were treated with keratocyte differentiation medium (KDM) consisting of Advanced DMEM (Gibco 12491, Invitrogen) including 400.0 mg/L AlbuMAX® II and 10.0 mg/L insulin recombinant full chain, and supplemented with 1.0 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich), 2 mM L-alanyl-L-glutamine (Gibco GlutaMax™-1, Invitrogen), 50 µg/mL gentamicin (Invitrogen), 100 U/mL penicillin (Mediatech), 100µg/mL Streptomycin (Mediatech), 10 ng/mL basic fibroblast growth factor (FGF-2, Cell Sciences) and 0.1 ng/mL transforming growth factor-beta3 (TGF-β3, R&D Systems).(Wu et al. 2013) The medium was changed twice per week for up to 9 wk.

2.3 Two-photon fluorescence microscopy

Two-photon fluorescent micrographs were collected employing an Olympus FV 1000 multiphoton microscope (Center Valley, PA). To collect the second harmonic generation (SHG) signal from collagen fibrils, three-dimensional image sets were captured at a section spacing of 2 µm, using a 25 ×1.0 NA objective at a laser wavelength of 830 nm. Images were processed into three-dimensional stacks using Imaris (Bitplane).(Wu et al. 2012; Wu et al. 2013)

2.4 Electron microscopy

Specimens for electron microscopy were prepared as described previously. (Wu et al. 2012; Wu et al. 2013) Briefly, all of the specimens were fixed in cold 2.5% glutaraldehyde (EM

grade, Taab Chemical) in 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), and dehydrated through a graded series of ethanol washes. For scanning electron microscopy (SEM), they were further treated with a hexamethyldisilazane wash. The yielded samples were imaged with a Jeol JSM-6330F Scanning Electron Microscope (JEOL Ltd.) working at 5 kV. For transmission electron microscopy (TEM), the dehydrated samples were embedded and cured in Epon (Energy Beam Sciences). Sections were cut perpendicular to the alignment of the underlying grooved substrates. Semi-thin sections (300 nm) were stained with 0.5% Toluidine Blue (Fisher) and examined under optical microscopy (Elipse E600, Nikon). Ultra-thin sections (65nm) were stained with 2% uranyl acetate (Electron Microscopy Sciences) in a 1:1 mixture of water and methanol, and then with aqueous 1% phosphotungstic acid (Sigma-Aldrich), pH 3.2. The sections were examined and photographed with a Jeol 1011 transmission electron microscope (JEOL Ltd.) working at 80 kV.

2.5 Gene expression

Quantitative RT-PCR (qPCR) was carried out to examine gene expression of hCSSCs and hCFs after KDM treatment, as described previously.(Wu et al. 2012) DNase-treated total RNA (400 ng) isolated from constructs by the RNeasy mini kit (Qiagen) was reversed-transcribed to cDNA by Super Script III (Invitrogen) in the presence of random hexamers (Invitrogen). Quantitative PCR (qPCR) of cDNA equivalent to 20 ng RNA was performed with direct dye binding (SYBR Green; Fermentas and Fisher) in a StepOne Plus System (Applied Biosystems) according to the manufacturer's instruction. Technical triplicate samples were run for each reaction. A dissociation curve for each SYBR-based reaction was generated to confirm that there was no nonspecific amplification. Amplification of 18S rRNA was conducted for each cDNA for normalization of RNA content. Relative mRNA abundance was calculated as the cycle threshold (Ct) for amplification of a gene-specific cDNA minus the average Ct for 18S expressed as a power of 2 ($2^{-\Delta Ct}$). Three individual gene-specific values thus calculated were averaged to obtain a mean $\pm$ SD. Primer sequences were previously published.(Du et al. 2007)

2.6 Whole-mount immunostaining

After culture in stem cell growth medium (SCGM) for 3 days, morphology and confluence of viable hCSSCs and hCFs seeded on aligned PEUU fibrous substrates were evaluated by fluorescent Calcein AM (Invitrogen) staining. Briefly, the samples were rinsed by PBS solution three times (5 min each), followed by addition of 2 μ M Calcein AM working solution and stored in a standard incubator (5% CO₂, 37°C). After 30 min, the samples were gently rinsed with PBS, then placed in a Delta TPG Culture Dish (0.17mm thick glass, Bioptechs) and examined using an Olympus FluoView FV1000 confocal microscope.

After 9 wk culture in KDM, the ECMs deposited by hCSSCs and hCFs on highly aligned PEUU substrates were fixed in a 2.5% paraformaldehyde (PFA, Electron Microscopy Science) PBS solution at room temperature for 20 min, then rinsed in PBS three times (10 min each time), and stored at 4°C in PBS for further processing. Except for the case of keratocan staining, non-specific binding of the fixed samples was blocked by 10% heat-inactivated goat serum at room temperature for 1 h. After that, the samples were rinsed in PBS and incubated with mouse-monoclonal primary antibodies diluted with 1% bovine serum albumin (BSA, Fluka) in PBS overnight at 4°C in a sealed moist box. For immunostaining of keratocan, the samples were first digested with keratanase (0.5 U/mL, Sigma Aldrich) in 1% BSA in PBS for 2 h at 37°C, rinsed in PBS three times (10 min each), and then incubated with primary goat anti-human keratocan antibody (Kera C, a gift from

Winston Kao, the University of Cincinnati, Cincinnati OH) overnight at 4°C, followed by three washes (10 min each) in PBS.

For myofibroblast staining, fixed cells were first permeabilized with 0.1% Triton X-100 in PBS for 20 min, and thoroughly washed by PBS. The permeabilized cells were labeled with Alexa Fluor 488 phalloidin (2 µg/ml, Invitrogen), and incubated with mouse anti-human α -SMA (1:100, Sigma-Aldrich) overnight at 4°C, then washed with PBS three times (10 min each).

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

2.7 Western blotting

Proteoglycans were isolated and collected from the culture medium employing SPEC 3 NH₂-ion exchange columns (Agilent Technologies), dialyzed, and dried as previously described (Funderburgh et al. 2003). Portions of each sample were digested by 0.1 U/mL chondroitinase ABC (Sigma-Aldrich) or 0.5 U/mL keratanase (Sigma-Aldrich) overnight at 37°C in 0.1 M ammonium acetate, pH 7.5. Digested samples were run on 4–20% SDS-PAGE gels (Bio-Rad Laboratories), then transferred to PVDF-FL membrane (Millipore) and detected using immunoblot analysis with specific antibodies. Keratocan was identified with antibody Kera C (provided by Winston Kao, University of Cincinnati, Cincinnati OH), lumican with monoclonal antibody Lum-1 (provided by Bruce Caterson, Cardiff University, Wales, UK) (Young et al. 2009), decorin with polyclonal anti-DCN antibody (Sigma-Aldrich), keratan sulfate with monoclonal antibody J19 (a gift from Nirmala SundarRaj, the University of Pittsburgh, Pittsburgh, PA) and dermatan sulfate with monoclonal antibody BE123 (Millipore). Primary antibodies were detected with IR dye-labeled secondary antibody (LI-COR Biosciences), and visualized using the LiCor Odyssey Imager. The loading for the detection of keratocan and lumican is half of others.

To re-probe decorin on each blot, all of these tested membranes (except the one for decorin) were stripped using Western Restore stripping buffer (Pierce) for 1 hour at room temperature. The stripped membranes were rinsed in PBS with 0.1% Tween-20 three times for 10 min each, and then digested overnight at 37 °C in Chondroitinase ABC Buffer with 0.1 U/mL chondroitinase ABC (Sigma-Aldrich) and 1% bovine serum albumin. The membrane was then treated with anti-DCN antibody.

2.8 Statistical analysis

One way ANOVA followed by *post-hoc* Newman-Keuls multiple comparison testing was employed to evaluate gene expression of hCSSCs and hCFs after their differentiation in KDM culture. Significance was considered to exist at $p < 0.05$. All results are presented as mean $\pm$ standard deviation.

3. Results

3.1 Cell morphology on highly aligned PEUU fibrous substrates

As shown in Fig 1, the aligned PEUU fibrous substrate (Fig. 1a) provided orientation guidance to induce both hCSSCs (Fig. 1b) and hCFs (Fig. 1c) to elongate and align in the preferred direction of the fibrous substrate. Morphologically, hCFs appeared larger, more

elongated and spindle-like than hCSCCs. Both hCSCCs and hCFs were confluent after 3 days culture.

3.2 Cell phenotype

After reaching confluence, hCSCCs and hCFs were switched to serum-free keratocyte differentiation medium (KDM) including FGF-2 and TGF- β 3 supplementation. The differentiated hCSCCs on aligned PEUU fibrous substrate showed F-actin fibril bundles generally aligned with the major cell axis (Fig. 2a) and weak fluorescent expression of α -SMA (Fig. 2c). The aligned hCFs also showed pronounced F-actin fibrils in alignment with the underlying fiber direction (Fig. 2b), but the intra-cellular α -SMA expression in hCFs (Fig. 2d) was much stronger than for hCSCCs (Fig. 2c). No fluorescence was observed from the PEUU substrate alone at the imaging settings used for the other images in Fig. 2.

3.3 Gene expression of hCSCCs and hCFs

Gene expression of hCSCCs and hCFs differentiated in KDM was examined by qPCR. Fig. 3 shows the gene expression patterns of differentiated hCSCCs and hCFs normalized to 18S. Before KDM treatment, hCSCCs had hardly detectable expression of typical gene markers for keratocytes including KERA, CHST6 and B3GNT7. After differentiation in KDM, hCSCCs substantially up-regulated these typical markers, in agreement with previous reports. (Wu et al. 2012; Chan et al. 2013; Wu et al. 2013) In addition, EDA-Fn and α -SMA, two typical myofibroblast markers, had low expression levels that were comparable to those for hCSCCs cultured in SCGM.

Like hCSCCs, hCFs initially had nearly undetectable expression of KERA and B3GNT7 and very low expression of CHST6 before KDM treatment. After culture in KDM for 9 wk, hCFs similarly experienced an up-regulation in gene expression characteristic of keratocytes. However, the expression levels of these genes were significantly lower than for those of differentiated hCSCCs. Meanwhile, the expression levels for myofibroblast characteristic genes (i.e. EDA-Fn and α -SMA) were up-regulated and significantly higher than for hCSCCs.

3.4 Expression of extracellular matrix (ECM) components by hCSCCs and hCFs

As shown in Fig 4a–c, the ECM secreted by hCSCCs was abundant in collagen type-I (Col-I), collagen type-V (Col-V) and collagen type-VI (Col-VI), typical collagen components in human corneal stromal tissue. The ECM deposited by hCSCCs was homogeneous and densely populated with collagens on the aligned PEUU fibrous substrate. Compared with hCSCCs [Fig. 4(a–e)], hCFs exhibited much lower potential to generate collagen fibril-based ECM [Fig. 4(f–j)]. As shown in Fig. 4f, collagen type-I fibrils deposited by hCFs were sparsely distributed on the aligned PEUU substrate, though maintaining a high degree of alignment. The expression of two other key collagen components, collagen type-V and type-VI (Fig. 4g, h), was weak and not comparable to those deposited by hCSCCs (Fig. 4b, c). For cornea-specific proteoglycans, ECM secreted by hCSCCs showed clear expression of keratan sulfate and keratocan (Fig. 4c, d). In contrast, these components were not readily detected in hCF-secreted ECM (Fig. 4i, j).

3.5 Sulfate proteoglycans recovered from culture media

Keratan sulfate proteoglycan (KSPG) and dermatan sulfate proteoglycan (DSPG) are two typical proteoglycans in the native corneal stroma. Specially, KSPG and its core proteins, including keratocan, lumican and mimican, are uniquely present in the human cornea and representative markers of differentiated tissue. During culture in KDM, these secreted proteoglycans could accumulate in the culture medium in addition to being incorporated in

the forming corneal stromal tissue. In Fig. 5 and Fig. 6, the time course of expression of KSPG and DSPG secreted by hCSSCs and hCFs is presented. The characteristic broad molecular weight band of keratan sulfate between 100 and 200 kDa can be seen in the samples from hCSSC culture. (Fig. 5a). In contrast, hCFs showed negligible expression of KSPG over the entire time course (Fig. 5b). After enzymatic digestion, the two core proteins of KSPG, lumican and keratocan, exhibited much stronger expression in hCSSC samples (Fig. 5c, e) versus hCF ones (Fig. 5d, f), and increased with culture time. The cell type dependence of the secretion of KSPG was consistent with the observations of gene expression and immunostaining shown in Fig. 3 and Fig. 4.

When dermatan sulfate was examined, two bands of distinct molecular weight can be detected with no obvious time dependence for either cell type (Fig. 6a, b). However, hCSSCs appeared to have lower dermatan sulfate expression levels (Fig. 6a) than hCFs (Fig. 6b). Decorin, one of the core proteins of DSPG, can be detected in both hCSSC and hCF samples (Fig. 6c, d). Interestingly, the expression of decorin had no distinct differences between cell types, and also did not appear to vary temporally. These results suggest that decorin expression is stable and in each proteoglycan sample can serve as an internal control. Accordingly, we stripped blots in Fig. 5 and Fig. 6 (except the one for decorin) and reprobed them with antibody to decorin. As shown in Fig. 5a'–b', Fig. 5c'–d' and Fig. 6a'–b', the expression of the reprobed decorin for each pair of samples was independent of cell type and varied little with time, indicating the variation of keratan sulfate markers was not a technical artifact.

3.6 Ultrastructure of collagenous ECM synthesized by hCSSCs and hCFs

As shown in Fig. 7a, ECM deposited by hCSSCs on highly aligned PEUU substrates generated a very strong second harmonic signal throughout the whole construct. Specifically, the projected second harmonic image implied that the collagen fibril-based ECM featured orthogonal orientation, in accordance with confocal fluorescent imaging (Fig. 4a). Similarly, ECM deposited by hCFs generated a second harmonic signal with aligned features, but the expression was much lower and less sparse, as well as lacking in orthogonal orientation (Fig. 7b).

Employing SEM, surface cell morphology and elaborated ECMs secreted by hCSSCs and hCFs were visualized (Fig. 8). On the highly aligned PEUU substrate, hCSSCs were elongated and uniformly aligned in one preferred direction (Fig. 8a). Similarly, confluent hCFs were stretched and parallel to each other with high fidelity (Fig. 8c). hCSSCs appeared to have a more flattened morphology than hCFs.

Viewing the top layer of cell-secreted ECM by SEM, differences are seen between ECM secreted by the hCSSCs (Fig. 8b) and that from hCFs (Fig. 8d). Higher magnification revealed that hCSSC-secreted collagen-fibril ECM was multi-layered and orthogonally oriented (Fig. 8b). In accordance with hCF alignment, many dense, aligned collagen fibrils were seen to be distributed between hCFs on the substrate. However, these generally aligned collagen fibrils lacked the degree and scale of orthogonally oriented layers found with hCSSCs.

Since visualization by SEM was limited at the top layer of cell-secreted ECM, optical microscopy and TEM were employed to further evaluate ECM ultrastructure and collagen fibril organization (Fig. 9). First, semi-thin cross sections (300 nm) of the ECMs secreted by hCSSCs (Fig. 9a) and hCFs (Fig. 9b) were evaluated by optical microscopy. In general, both collagen-fibril constructs were sandwiched by confluent and aligned hCSSC and hCF layers, respectively. Notably, hCSSCs showed much more compressed and flat (oblate) morphology than hCFs, which was consistent with observations under SEM. hCSSC-

secreted constructs were 60~70 μm thick, about 2 ~ 3 fold larger than for those secreted by hCFs.

For the thin sections (65 nm) visualized by TEM, the collagen fibril-based construct secreted by hCSCs presented stratified lamellae with orthogonal orientation (Fig. 9c): Collagen fibrils were alternately parallel to and normal to the viewing plane between the adjacent lamellae. In dense clusters, the fibrils were of uniform size, and the corresponding inter-fibril spacing exhibited long range regularity. On the contrary, hCFs secreted ECM is less abundant and less densely packed. The resultant sparse collagen fibrils did not organize well into the characteristic stratified lamellae and were lacking the regular inter-fibril spacing, characteristic of native corneal stromal tissue (Fig. 9d).

4. Discussion

Initial efforts to generate human corneal stromal tissue in vitro over the past several decades met with limited success.(Orwin and Hubel 2000; Li et al. 2003; Orwin et al. 2003; Crabb et al. 2006; Ren et al. 2008). More recently, however, two distinct experimental systems have reported the deposition of a multilamellar collagenous construct resembling the tissue of the corneal stroma. Studies from our laboratory have employed hCSCs in a serum-free culture medium using a biodegradable polyurethane fibrous substrate.(Wu et al. 2012; Wu et al. 2013) The alignment of the substratum was found to present topographical cues essential for the organization of the ECM by these cells. (Wu et al. 2012; Wu et al. 2013) Additionally, the initial elastic modulus of the PEUU fibrous substrate is 8 ± 2 MPa (Stankus et al. 2004), which is very close to the elastic modulus of human cornea (i.e. 3~13 MPa, Jue et al, 1986) and markedly different from tissue culture polystyrene. Whether the mechanical properties of the substratum plays a role in initiating differentiation is speculative at this point. Addition of FGF-2 and TGF- $\beta 3$ enhanced the effects of the geographical cues, allowing generation of a corneal stroma-like tissue: multilayered lamellae with orthogonally-oriented collagen fibrils and abundance in cornea-specific proteins and proteoglycans.(Wu et al. 2013) In a distinctly different experimental protocol, Zieske and collaborators found hCFs in serum-containing medium to generate similarly organized stromal-ECM when cultured on transwell membranes (Guo et al. 2007; Ren et al. 2008; Karamichos et al. 2010; Karamichos et al. 2011; Karamichos et al. 2012). These constructs were enhanced in organization by ascorbate analogs and by TGF- $\beta 3$.

Because the topography of the substratum plays such a clear role in stimulating ECM by hCSCs, in the current report, we sought to compare these cells with corneal fibroblasts (hCFs) on a highly aligned PEUU substrate to elucidate the role of the substratum in organizing a stromal matrix. In this study, we also used the previously reported serum-free medium containing insulin and albumin and ascorbate analogs. We also examined the effects of supplementation with FGF-2 and TGF- $\beta 3$ to induce keratocyte differentiation and ECM production in hCSCs and hCFs.

The data confirmed previous observations that hCSCs cultured on a highly-aligned fibrous substrate differentiated into cells with gene expression profiles similar to those of human keratocytes and deposited highly-organized collagen that was compositionally and ultrastructurally similar to that of human corneal stroma. In the presence of FGF-2 and TGF- $\beta 3$, hCFs exhibited clear differences from the hCSCs. These cells did up-regulate expression of keratocyte marker genes (Fig. 3), but not to the extent that hCSCs did. On the other hand, expression levels of Fn-EDA and α -SMA, markers of myofibroblasts, were also substantially up-regulated in hCFs. The expression of these two pathologic markers was much lower in hCSCs. Thus, with respect to gene expression, it appears that hCFs can be

partially recovered to keratocytes, but also have a tendency toward myofibroblast differentiation under the conditions of this study.

The assay of ECM deposition and organization further pointed out differences in these two cell types under equivalent culture conditions. As shown in Fig. 4, collagen types I, V, and VI, the major protein components stromal tissue, were much less abundant in the hCF-secreted constructs than in those produced by hCSSCs. Furthermore, ECM secreted by hCFs was lacking in cornea-specific keratan sulfate proteoglycans, keratocan and lumican, which are critical to regulate inter-fibril spacing (Funderburgh et al. 1986; Chakravarti et al. 1998; Funderburgh 2000; Kao and Liu 2002; Carlson et al. 2005; Chakravarti et al. 2006). In contrast, hCSSC-secreted ECM was abundant in collagen types I, V and VI as well as keratan sulfate proteoglycans, keratocan and lumican. (Fig. 5).

Accordingly, hCSSCs-secreted ECM is akin to native human corneal stromal tissue, characteristic of multi-layered collagen fibril lamella with orthogonal orientation and uniform fibril size and interfibril spacing. By comparison, hCF-secreted ECM exhibits characteristics of human corneal scar tissue, in which collagen fibrils lack the hierarchical organization of human corneal stromal and inter-fibril spacing is irregular. These defects stem from the pathogenic differentiation from keratocytes to myofibroblasts, where the expression of keratocan in native keratocytes is lacking and high expression levels of α -SMA and stress-induced F-actin fibril bundles occur.

In previous studies by Zieske and co-workers (Guo et al. 2007; Ren et al. 2008; Karamichos et al. 2010; Karamichos et al. 2011; Karamichos et al. 2012), hCFs were found to produce a stromal-like ECM similar in thickness and organization to that reported for hCSSCs. (Wu et al. 2012; Wu et al. 2013) In this direct comparison, the hCFs did not match hCSSCs in production of this matrix. These differences might arise from several sources. The hCFs used in our current study were obtained from collagenase digestion of whole stromal tissue, whereas the studies of Zieske and colleagues used cells that grew out from stromal tissue explants. These differing methods may have selected for cells with distinct differentiation potentials. Also, the previous hCF studies were carried out in serum-containing medium whereas the conditions here were serum free. The possibility cannot be excluded that serum contains components required for fibroblast differentiation that are not required by hCSSCs. Such a requirement could represent a disadvantage to the use of fibroblasts in corneal stromal tissue engineering. High levels of serum are likely only to be available from xenogenic or allogenic sources and will thus render constructs for human use much more expensive and pose potential regulatory hurdles. A related disadvantage of using hCFs is the extent to which these cells can be expanded before they are used to produce keratocytes. Stem cells are known to exhibit an extended cellular lifespan, and previously we reported that progenitor cells from bovine stroma can be passaged through at least 50 population doublings while maintaining potential to differentiate to keratocytes.(Funderburgh et al. 2005) In contrast, unfractionated stromal cells lose the potential for expression of keratan sulfate after 5–7 passages.(Long et al. 2000)

Even though hCFs did not perform as well as hCSSCs at secreting and organizing stroma-like ECM under the conditions of this study, it was clear that aligned nano-fiber substratum did support differentiation of hCFs towards the keratocyte phenotype and allowed production of an organized ECM. Since hCF cells do not differentiate well on tissue culture plastic, these findings support our hypothesis that topographical cues are essential for inducing a stromal ECM in cells from the corneal stroma. This idea is supported by studies by Saeidi et al (Saeidi et al. 2012) using human corneal fibroblasts on a disorganized collagen substrata. hCFs also respond differently than dermal fibroblasts to substratum surface features in organizing matrix(Guillemette et al. 2009). Previous studies used a

commercial polycarbonate transwell membrane for support of the differentiating hCFs. (Guo et al. 2007; Ren et al. 2008; Karamichos et al. 2010; Karamichos et al. 2011; Karamichos et al. 2012) It remains to be determined in future studies how the surface architecture of transwell membranes and the aligned PEUU nanofibers might both provide similar information to corneal cells, but because of the difference in chemical composition of these materials, we hypothesize that the cues seem likely to be topographical rather than chemical.

5. Conclusions

On aligned fibrous substrates made from biodegradable PEUU, hCSCs could be induced to secrete and organize a type-I collagen-based ECM abundant in characteristic human corneal stromal ECM components. Spatial self-organization of the collagen-based ECM by hCSCs featured stratified multilayered collagen-fibril lamellae with orthogonal orientation, and uniform fibril size and inter-fibril spacing in a pattern mimicking human corneal stromal tissue. Under the same culture conditions, ECM secreted by hCFs lacked cornea-specific ECM components, leading to a less-organized ultrastructure in a pattern akin to corneal scar-tissue. These observations demonstrated the potential for hCSCs, under proper substrate and growth factor guidance, to facilitate the generation of a biological human cornea equivalent. In contrast, hCFs were not similarly responsive to these environmental cues and generated an ECM that poorly mimicked native, functional tissue structure and composition, thus emphasizing the critical importance of cell type in pursuing corneal stromal engineering.

Acknowledgments

The authors would like to thank Dr. Simon C. Watkins, Mr. Gregory Gibson and Mrs. Ming Sun from Center of Biologic Imaging (CBI) of University of Pittsburgh for their support and assistance in two-photon microscopy and sample preparation for transmission electron microscopy (TEM). This work was supported by NIH grants EY016415 to J.L. Funderburgh, and Core grant P30-EY08098. Other support was received from the Ocular Tissue Engineering and Regenerative Ophthalmology (OTERO) program of the Louis J Fox Center for Vision Restoration, Research to Prevent Blindness Inc, the McGowan Institute for Regenerative Medicine.

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Highlights

- Corneal stromal stem cells have greater potential to generate stromal-like tissue.
- Matrix generated by corneal fibroblasts is not well-organized.
- Stem cell-based constructs exhibit more abundant corneal proteoglycans.
- Stem cell-based constructs present more corneal collagen-type I, V, VI.

Fig. 1. Scanning electronic micrograph of (a) aligned poly(ester urethane) urea (PEUU) fibrous substrate and confocal laser-scanning micrographs of cell response at 3 days to the aligned PEUU substrates: (b) human corneal stromal stem cells (hCSCs) and (c) human corneal fibroblasts (hCFs). The cells were stained by Calcein AM.

Fig. 2. Comparison of cytoskeletal reorganization (F-actin (green), a, d) and expression of α -SMA (red, b, e) of hCSSCs (a, b) and hCFs (d, e) with the 9-week treatment of keratocyte differentiation medium (KDM). Nuclei were stained by DAPI (blue): (c) hCSSCs and (f) hCFs.

Fig. 3.

Comparison of gene expression profiles for hCSCs and hCFs before (black) and after (grey) treatment with keratocyte differentiation medium (KDM). mRNA levels for each transcript were relative to 18S. Error bars show SD of three independent samples. For each gene, expression levels after KDM treatment were significantly different between the studied cell lines (*p < 0.05).

Fig. 4. Immunofluorescent micrographs of hCSSCs-secreted (a–e) and hCFs-secreted (f–j) corneal stroma-specific collagens and proteoglycans on aligned PEUU fibrous substrates: (a, f) collagen I, (b, g) collagen V, (c, h) collagen VI, (d, i) keratan sulfate and (e, j) keratocan. Nuclei were stained by DAPI (blue).

Fig. 5.

Western blots of corneal keratan sulfate proteoglycans (a, b), lumican (c, d) and keratocan (e, f) recovered from culture media of hCSSCs (a, c, e) and hCFs (b, d, f). For each cell type, lumican and keratocan were detected in the same blotting membrane by double-labeling. Reprobed decorin in the same blots (a, b, c/e, d/f) were shown in (a', b', c', d').

Fig. 6. Western blots of corneal dermatan sulfate proteoglycans (a, b) and decorin (c, d) recovered from culture media of hCSSCs (a, c) and hCFs (b, d). Re-probed decorin in the blots (a, b) are shown in (a') and (b').

Fig. 7. Two-photon fluorescent micrographs of ECMs secreted by (a, c, e) hCSSCs and (b, d, f) hCFs: (a, b) SHG signals from collagens, (c, d) SHG signals from auto-fluorescence of hCSSCs and hCFs, and (e, f) merged images.

Fig. 8. SEM micrographs of ECMs secreted by (a, b) hCSCs and (c, d) hCFs on the aligned PEUU fibrous substrates at 9 wk.

Fig. 9. Semi-thin cross sections of ECM secreted by (a) hCSSCs and (b) hCFs were stained with toluidine blue and examined by optical microscopy to demonstrate cell shape and location, and construct thickness. The ultrastructures of the elaborated ECM from (c) hCSSCs and (d) hCFs were evaluated by transmission electron microscope (TEM).