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Collagen fibril diameter and alignment promote the quiescent keratocyte phenotype

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Abstract

In this study, we investigated how matrix nanotopography affects corneal fibroblast phenotype and matrix synthesis. To this end, corneal fibroblasts isolated from bovine corneas were grown on collagen nanofiber scaffolds of different diameters and alignment – 30 nm aligned fibrils (30A), 300 nm or larger aligned fibrils (300A), and 30 nm nonaligned fibrils (30NA) in comparison to collagen coated flat glass substrates (FC). Cell morphology was visualized using confocal microscopy. Quantitative PCR was used to measure expression levels of six target genes: the corneal crystallin - transketolase (TKT), the myofibroblast marker - α -smooth muscle actin (SMA), and four matrix proteins - collagen 1 (COL1), collagen 3 (COL3), fibronectin (FN) and biglycan. It was found that SMA expression was down-regulated and TKT expression was increased on all three collagen nanofiber substrates, compared to the FC control substrates. However, COL3 and biglycan expression was also significantly increased on 300A, compared to the FC substrates. Thus matrix nanotopography down-regulates the fibrotic phenotype, promotes formation of the quiescent keratocyte phenotype and influences matrix synthesis. These results have significant implications for the engineering of corneal replacements and for promoting regenerative healing of the cornea after disease and/or injury.

Keywords

Cornea; keratocyte; fibroblast; nanotopography; tissue engineering

Introduction

Corneal disease is a major cause of blindness affecting more than 10 million people worldwide.¹ Corneal transplants are currently the only available treatment to restore vision, with approximately 40,000 transplants being performed in the United States each year.² Shortcomings of the procedure include immune rejections, infections and donor shortages. This has motivated several attempts to develop tissue engineered corneal replacements.^{3,4,5} The cornea has three main layers – the epithelium, endothelium and stroma.¹ The stroma

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contains quiescent cells called keratocytes that reside within uniformly aligned collagen fibrils, 30 – 35 nm in diameter (see Supplementary Material). Uniformity of fibril diameter and alignment are thought to be instrumental in maintaining transparency of the cornea. The fibrils are further arranged in alternating arrays called lamellae (1 – 2 μ m in thickness) which are stacked orthogonally leading to a lattice like arrangement which provides mechanical strength to the cornea. The difficulty in recreating the organized nanoscale architecture of the stroma has been one of the primary impediments to the successful production of a tissue engineered corneal replacement.

When the cornea sustains injury such as an abrasion which disrupts the extracellular matrix (ECM), the quiescent corneal keratocytes adjacent to the wound undergo apoptosis.⁶ Keratocytes adjacent to the apoptotic region transform into repair cells including activated fibroblasts and myofibroblasts, the latter of which are characterized by the expression of α -smooth muscle actin (SMA). These cells invade the wound region and attempt to repair the injury site by secreting matrix proteins such as collagen 1 (COL1), collagen 3 (COL3), fibronectin (FN) and biglycan. However, the matrix proteins that are secreted form a disorganized matrix of irregular scar tissue with decreased transparency. This decrease in transparency is exacerbated by the loss of corneal crystallins, such as transketolase (TKT). Corneal crystallins are responsible for index-matching the cell body of the keratocyte with the stroma and improving light transmission through the cells.⁷ Quiescent cells express corneal crystallins which improve the transparency of the cornea, while activated cells such as myofibroblasts have reduced expression of crystallin proteins. Interestingly, in the case of corneal burns or freeze injuries where the ECM is left intact, quiescent corneal keratocytes proliferate and repopulate the wound without phenotype switching and without scar formation.⁸ Thus the integrity of the matrix and the nanoscale arrangement of the collagen fibrils seem to be important for regenerative healing of the cornea after injury.

Previous studies have shown that corneal fibroblasts align and migrate differentially on substrates with different topographical features.^{9,10} Phu *et al.* have shown that corneal fibroblasts cultured on aligned collagen fibers down-regulate SMA compared to cells grown on unaligned fibers or tissue culture polystyrene (TCPS).² However, the effect of fibril diameter or alignment on the expression of corneal crystallins and matrix proteins has not been investigated thus far. The objective of this study was to evaluate how the topography of collagen fibrils that keratocytes encounter *in vivo* affects cell phenotype, matrix synthesis, and potentially corneal wound healing after injury. We examined the individual contribution of two properties of the collagen fibrils - namely, fibril diameter and alignment, and their effect on cell phenotype and matrix production. To this end, three types of nanofiber scaffolds with collagen fibrils of varying topography - 30 nm aligned fibrils (30A), 300 nm or larger, aligned fibrils (300A), and 30 nm nonaligned fibrils (30NA), were assembled on glass substrates using a nanoweaving technology developed by Fibralign Corporation. The 30A substrates were selected as a representative arrangement of collagen fibrils *in vivo*. The 300A substrates were chosen to study the effect of fibril diameter, while the 30NA substrates were selected to investigate the effect of fibril alignment on cell response, respectively. Flat glass substrates coated with bovine collagen, type I (FC) were used as controls that present no predetermined topography to the cells. Bovine corneal fibroblasts were cultured on three nanofiber scaffolds (30A, 300A and 30NA) and the flat collagen coated controls. Cell morphology of the corneal fibroblasts was visualized by staining for f-actin and the nucleus using spectral confocal microscopy. Gene expression levels of six proteins – TKT, SMA, collagens 1 and 3, fibronectin, and biglycan were measured to evaluate the effect of nanotopography on matrix synthesis and cell phenotype.

Materials and methods

Corneal fibroblast isolation and cell culture

Bovine corneal fibroblast isolation was performed based on a previously published protocol.¹¹ Corneal buttons were isolated from bovine eyes (Manna Foods, Oakland, CA, USA) by making incisions around the corneal while excluding the scleral and limbal regions. The posterior surface of the buttons was scraped to remove endothelial cells. Buttons were then immersed in a Dispase I solution at 100 mg/ml (Roche, Indianapolis, IN, USA) for four hours and epithelial cells were removed using repeated scalpel strokes while the buttons were still immersed in the Dispase solution. After rinsing with phosphate buffered saline (PBS), the corneal buttons were cut in half and placed on a fibronectin coated plate. Fibronectin was obtained from Athena Environmental Sciences (Baltimore, MD, USA) and plates were coated according to the manufacturer's instructions. The corneal buttons were then covered with corneal fibroblast complete media (DMEM + 10% fetal bovine serum, FBS + 1% Penicillin-Streptomycin solution + 100 mg/L sodium pyruvate), where FBS is an activator of the corneal fibroblast phenotype.¹² The corneas were then incubated at 37 °C and 5% CO₂ until the plates were 85% confluent. Then, the corneal fibroblasts were harvested using trypsin (2.5g/L in 2.5 mM EDTA) and resuspended in complete media. Corneal fibroblasts were cultured in fibronectin coated tissue culture flasks in complete media that was changed three times a week. Cells were split approximately once or twice a week and cultured in a humidified 95% air/5% CO₂ incubator at 37°C. The cells used in these experiments were passaged less than 5 times.

Collagen nanofiber scaffolds

The crimped fibril configuration (Figure 1A) is typical for collagen-based fibrous tissue not under an external load. There are two basic models of crimp collagen fibrils: planar wave/zig-zag model and cylindrical helix model¹³ (Figure 1B), but neither of these can explain the double crimp pattern seen in Figure 1A. This structure is formed by combining the left-handed and right-handed helical fibrils into a double super helix structure. The fibrils nest together to create a crimp pattern that is formed by the bunching of adjacent fibrils. This model¹⁴ is schematically represented in Figure 1C. The green fibrils are left-handed while the red fibrils are right-handed. The actual crimps are the cluster of peaks of the nested helices.

The collagen nanofiber scaffolds with the double super helix model described above were fabricated accordingly to previously published protocols.^{15,16} The production process is based on technology developed for liquid crystal display (LCD) manufacturing^{17,18,19,20,21} and is suitable for lyotropic liquid crystal materials. Purified monomeric collagen solution (bovine type I, 3 mg/ml) was purchased from Advanced BioMatrix (San Diego, CA, USA). This material was concentrated to reach a liquid crystal state and sheared onto glass with optical precision using a liquid film applicator assembly.²² A second generation machine using air-bearing systems (Figures 2A, 2B) provides optimal conditions for the self-assembly of collagen molecules into aligned and aligned-braided fibrils. The glass substrate is mounted on a stage which is transported by a linear actuator under a stationary applicator head. The head was adjusted to produce collagen matrices of different coating thicknesses and required crimp structures with coating speeds of 100 mm/sec using small quantities (1–2 ml) of collagen solution at typical concentrations of 25 mg/ml. A representative image of the scaffold with 300 nm aligned collagen fibrils (300A) is shown in Figure 2C. Fiber diameter and alignment were varied by changing pH, collagen concentration, humidity and other factors.

For the fabrication of the unstructured collagen coated slides, flat glass substrates were rinsed in PBS and deionized water and then sterilized in 70% ethanol and covered with bovine collagen I (BD Biosciences) at a concentration of 50 µg/ml in 0.01 M HCl using a pipette and incubated at room temperature for one hour. Excess collagen solution was then aspirated and the slides were rinsed with PBS. Substrates were either used immediately or stored in 70% ethanol at 2 – 8 °C for up to two weeks before use.

Atomic force microscopy and diffraction

Samples were air dried before imaging using atomic force microscopy (AFM). The structure of the collagen fibrils on the glass substrate was observed using AFM Ntegra Prima and Solver Next (NT-MDT, Moscow, Russia). Images were acquired in the semi-contact mode using silicon tips NSG01 with typical radius < 10 nm and spring constant 5.1 N/m (K-Tek Nanotechnology, Wilsonville, OR, USA). The diffraction patterns were produced using a red (630nm) laser beam focused to a diameter of about 0.3 mm on the collagen layers deposited on glass substrates.

Visible light transmission

The transmission of visible polarized light through the 30A collagen layer was measured using a LAMBDA 950 UV/Vis/NIT Spectrophotometer (Waltham, Massachusetts, USA). Spectra were measured through the substrate and bare glass was used as the baseline which was then subtracted to obtain transmittance through the collagen layer alone. S-direction was parallel to the crimp and p-direction was parallel to the direction of the fibrils.

Confocal microscopy

Collagen nanofiber substrates (30A, 300A and 30NA) and collagen coated flat glass controls (FC) were seeded with corneal fibroblasts at a density of 10,000 cells/cm² in complete media and allowed to grow for two days. Complete media was changed to media without FBS the day after seeding. After two days, the cells were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA, USA) for 15 min, permeabilized with 0.5% Triton X-100 (Sigma, St. Louis, MO, USA) for 5 min, and blocked with 1% bovine serum albumin (Sigma) for 30 min. F-actin was stained using Alexa Fluor 568 phalloidin (Invitrogen, Carlsbad, CA, USA) for 60 min. Nuclei were then counterstained with Hoechst 33258 for 5 min. All images were acquired using a Nikon C1si spectral confocal microscope.

Morphometric analysis

Cell shape index (CSI) is defined here as the dimensionless ratio: $4\pi * A * P^{-2}$, where A = cell area and P = cell perimeter.²³ CSI provides a measure of cell circularity with circular cells having values closer to one, and elongated cells having values approaching zero. CSI was calculated from the spectral confocal images. Four images were acquired per substrate to calculate CSI values.

Quantitative polymerase chain reaction

Bovine corneal fibroblasts were seeded on the collagen nanofiber scaffolds – 30A, 300A, 30NA and FC at a density of 10,000 cells/cm². Complete media was changed to media without FBS the day after seeding. Samples were harvested after two days using the Ambion/Applied Biosystems's Cell to C_T kit (Foster City, CA, USA). Lysis, RT-PCR, and qPCR were performed according to manufacturer's instructions using a StepOne Plus instrument (Applied Biosystems). The qPCR amplification protocol consisted of one cycle at 95 °C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. The relative amount of cDNA was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) present in each sample. Primers (Integrated DNA Technologies,

Coralville, IA, USA) are provided in Table 1. Melt curve analyses was performed at the end of all PCRs to ensure the specificity of PCR. Each experimental condition was performed three to five times. Expression levels of the genes were measured in technical triplicates.

Statistical analysis

Statistically significant differences were estimated using analysis of variance (ANOVA) tests. For group differences shown to be significant by ANOVA, sequential Holm t-tests were performed to detect differences between specific pairs of substrates. Differences were considered significant if $p < 0.05$.

Results and discussion

Characterization of nanofiber substrates

Collagen nanofiber scaffolds with the three different arrangements of collagen fibrils deposited on glass substrates (30A, 300A and 30NA) were fabricated by Fibralign Corporation. AFM analysis of the 30A substrate, mimicking native corneal fibrils, shows highly aligned 30 nm collagen fibrils (Figure 3A). The direction of crimp is also shown (green arrow). AFM imaging of 300A substrates showed thick aligned fibrils with a diameter of around 300 nm (Figure 3B), while the 30NA substrates showed nonaligned fibrils with a diameter of approximately 30 nm (Figure 3C). All samples were uniformly covered with the collagen fibrils. Fibril arrangement remains intact after seeding cells as shown by scanning electron microscopy (see Supplementary Material for representative images of 30A and FC substrates).

Diffraction patterns produced using a 630 nm laser beam were also used to assess the alignment of the fibrils. The two vertical petals and “cross” seen in the diffraction patterns in Figure 4A and 4B indicate a high alignment of the collagen fibrils on the 30A and 300A substrates. The elliptical diffraction area and high level of diffusive scattering in Figure 4C indicate a lesser alignment and poor periodicity of the crimp structure on the 30NA substrate.

The 30A substrate which mimics the fibril arrangement *in vivo* was further evaluated to measure the transmission of visible polarized light through the collagen layer. As seen in Figure 4D, the optical transmission through the collagen layer is high (~90%) in the visible region of the spectrum. The birefringence of the layer is consistent with the form birefringence which is related to the aligned fibrillar structure of the 30A substrate. The high optical transmission through the 30A substrate should be beneficial for the use of these collagen films as templates for corneal tissue engineering scaffolds in future studies.

Corneal fibroblast morphology on collagen nanofiber substrates

To determine how matrix nanotopography affects cell morphology, bovine corneal fibroblasts were seeded on 30A, 300A and 30NA substrates and on the flat collagen coated control, FC. After two days, cells were fixed and stained with phalloidin to visualize the actin cytoskeleton and Hoechst to image the cell nucleus using confocal microscopy. Representative images are shown in Figure 5. Cells grown on the 30A substrate were highly aligned and exhibited elongated and dendritic morphologies, similar to those of corneal keratocytes which represent the quiescent phenotype (Figure 5A, 5E).²⁴ Multiple cells had extended processes interconnecting with other cells. Cells on the 300A (Figure 5B, 5F) and 30NA (Figure 5C, 5G) were larger and more spread out with fewer processes extending out, while cells on the FC substrate were even more spread out with almost no extending processes as seen in Figure 5D and 5H. The cells that were spread out were similar in

appearance to repair phenotypes such as fibroblasts and myofibroblasts²⁴ with filamentous actin forming stress fibers across the cell body.

Morphometric analysis of the images obtained using confocal microscopy shows that there were significantly larger numbers of elongated cells on the nanofiber scaffolds (30A, 300A and 30NA) compared to the flat control, FC (Figure 6, * $p < 0.001$). Also, the 30A substrate has the maximum number of elongated cells significantly higher than those on any of the other substrates as seen in Figure 6 ($\Delta p < 0.001$). Thus corneal fibroblasts respond to the presence of the 30 nm, aligned collagen fibrils (30A) similar to those seen *in vivo*, by reverting to the stellate morphology exhibited by corneal keratocytes.

Previous studies with rabbit corneal fibroblasts have shown that the cells align themselves only on aligned collagen scaffolds and not on unaligned scaffolds or TCPS² in accordance with our results. Human corneal keratocytes cultured on micropatterned collagen also aligned along the direction of the grooves and secreted matrix proteins such as collagen 1 and keratan sulfate which appeared to be similarly aligned with the patterns.¹⁰ In another study, rabbit corneal fibroblasts were seeded on collagen coated patterned polyurethane substrates. It was shown that cells align themselves strongly on pitch sizes of 800 nm or larger and not on feature sizes of 400 nm.⁹ This is in agreement with our data, where cells do not align on collagen fibrils 300 nm or larger in diameter (300A). However, it is interesting to note that corneal fibroblasts respond to topography again when the feature size is further reduced to the 30 nm, aligned fibril arrangement similar to that seen *in vivo*. The corneal stroma is composed of collagen fibrils 30 – 35 nm in diameter, which are further arranged in orthogonal stacks called lamellae, which are 1 – 2 μm in thickness.¹ Thus the fact that the cells are able to respond to features on the order of microns (1 μm or larger, similar to lamellae), as well as down to nanometers (30 nm, similar to individual fibrils), could likely be influenced by the topography of the stromal fibrils and lamellae that the cells contact *in vivo*.

Target gene expression on collagen nanofiber substrates – 30A

Cell response was evaluated by qPCR for the following six markers – TKT, SMA, COL1, COL3, FN and biglycan. TKT is a corneal crystallin whose expression levels are elevated in the keratocyte phenotype⁷ and SMA is a marker for the fibrotic myofibroblast phenotype.²⁴ COL1, COL3, FN and biglycan are matrix proteins in the corneal stroma, all of which are up-regulated during scar formation.²⁴ We hypothesized that 30A substrates which mimic matrix topography *in vivo*, provide cues that decrease fibrosis and promote regeneration in corneal fibroblast cultures. As seen in Figure 7, qPCR analysis revealed a significant change in expression levels of TKT and SMA on 30A substrates compared to the flat controls, FC. When normalized by TKT expression on FC, corneal fibroblasts on 30A expressed 1.94 ± 0.51 times that of TKT levels on FC ($p < 0.05$). qPCR also showed a two-fold decrease in SMA expression on the 30A compared to FC. When normalized by expression on FC, SMA expression was 0.49 ± 0.11 fold decreased on 30A ($p < 0.05$).

Healthy corneal keratocytes are associated with abundant levels of TKT expression, and almost no expression of SMA.^{6,7} Thus it is seen that the presence of 30 nm, aligned collagen fibrils provides cues that promote the formation of the quiescent keratocyte phenotype by reducing SMA expression and increasing levels of TKT, which in turn, contributes to cellular transparency in the cornea. This data is in excellent agreement with our fluorescence microscopy results showing that maximal numbers of corneal fibroblasts assume an elongated, dendritic morphology resembling that of keratocytes on the 30A substrates compared to FC controls. Similar studies using rabbit corneal fibroblasts have shown that the percentage of fibroblasts expressing SMA is significantly reduced on scaffolds containing aligned collagen fibers when compared to cells grown on TCPS over a two-week

period.² Our study shows that this effect is seen as early as two days, and is accompanied by an increase in TKT gene expression. Previously it has been shown that poly(ethylene glycol) dimethacrylate hydrogel microrods in three-dimensional scaffolds significantly down-regulated SMA expression in 3T3 mouse fibroblasts, showing that topographical cues attenuate the expression of the fibrotic phenotype similar to our results.²⁵

While corneal fibroblast phenotype seemed to be sensitive to topography, COL1, COL3, FN and biglycan mRNA transcript levels on the 30A substrates were all statistically similar to FC controls (Figure 7). This suggests that matrix synthesis is not affected by the presence of 30 nm, aligned collagen fibrils. It has been shown that collagen synthesis in the corneal stroma is dependent on MEK kinase 1 (MEKK1), a member of the mitogen-activated protein kinase (MAPK) family.²⁶ Similarly, transforming growth factor-beta (TGF- β) mediated fibronectin synthesis occurs through a c-Jun N-terminal kinase dependent, Smad independent pathway in corneal stromal cells, while SMA expression requires signaling through Smad3.^{6,27} This could possibly explain why the effect of topography on the synthesis of matrix proteins such as fibronectin could be uncoupled from phenotype markers such as SMA.

Target gene expression on collagen nanofiber substrates – 300A

Fibril diameter is increased in scarred corneal tissue²⁸ leading to increased light scattering and loss of transparency. In order to examine the relative contribution of large fibril diameter on cell phenotype and matrix production, corneal fibroblasts were cultured on aligned collagen fibrils, 300 nm or larger in diameter (300A). Cell response was evaluated by qPCR analysis of the six targets listed above and mRNA expression was normalized to that of FC controls (Figure 7). Similar to the 30A substrates, TKT levels were increased and SMA levels were decreased on 300A substrates compared to FC. TKT expression on 300A substrates was 2.10 ± 0.78 times that of FC ($p < 0.05$), while SMA expression was 0.38 ± 0.13 times that of FC, when normalized to FC ($p < 0.05$). This suggests that fibril alignment has an effect on phenotype even at large diameters, as large fibrils when aligned, still promote the keratocyte phenotype (high TKT) and down-regulate the fibrotic phenotype (low SMA). This data is also in agreement with our fluorescence microscopy data which shows that there are more numbers of elongated cells (which resemble the keratocyte phenotype) on 300A substrates compared to FC controls.

However unlike the 30A scaffolds, qPCR analysis shows that COL3 and biglycan mRNA transcript levels were both significantly up-regulated in the presence of large fibrils when compared to flat controls (FC). COL3 expression was 2.83 ± 0.69 ($p < 0.05$) and biglycan expression was 2.34 ± 0.73 ($p < 0.05$) times respectively, on 300A compared to FC, when each was normalized to FC substrates. COL1 and FN levels were also increased on 300A compared to FC (2.47 ± 0.62 times for COL1, and 1.79 ± 0.68 times for FN, respectively) although not to statistically significant levels. Thus matrix synthesis is up-regulated in the presence of large diameter fibrils. There was no significant difference in gene expression levels between the 30A and 300A substrates.

Collagen fibrils in the normal stroma are very weak in scattering light because their diameter is smaller than the wavelength of light leading to a highly transparent cornea.¹ Collagen fibril diameter is significantly increased in corneal wound²⁹ and scar tissues.²⁸ It has been suggested that the enlargement in fibril diameter is responsible for the increase in light scattering associated with the scar tissue.²⁸ The increased synthesis of matrix proteins associated with large diameter fibrils could possibly lead to disorganized deposition of the matrix, which could explain the high scattering and reduced transparency observed in scar tissue.

Target gene expression on collagen nanofiber substrates – 30NA

Following injury, collagen fibrils in these regions of the cornea lose their highly ordered arrangement. Over time, the fibrils regain their alignment thus suggesting that aligned fibrils are important for proper wound repair.³⁰ Accordingly, corneal fibroblasts were cultured on 30 nm nonaligned collagen fibrils to examine the relative contribution of fibril alignment on cell phenotype and matrix production. Cell response was evaluated by qPCR analysis of the markers and matrix proteins listed above. As seen in Figure 7, TKT expression was significantly increased and SMA expression was significantly down-regulated on 30NA substrates compared to FC, similar to the results with the other two nanofiber scaffolds. TKT levels on 30NA substrates were 2.26 ± 0.26 times that of FC ($p < 0.05$), while SMA expression levels were 0.64 ± 0.18 times that of FC ($p < 0.05$). This suggests that cells still respond to collagen fibrils at the optimal diameter of 30 nm by up-regulation of the quiescent phenotype (high TKT) and down-regulating the repair phenotype (low SMA) even in the absence of complete alignment. This data is in accordance with our confocal microscopy data which shows that there are more numbers of elongated cells (similar to the keratocyte phenotype) on 30NA substrates compared to FC controls. As with the 30A substrates, mRNA transcript levels of all the matrix proteins tested -COL1, COL3, FN and biglycan, were statistically similar to that of the FC control. This suggests that matrix synthesis is not affected by the nonalignment of 30 nm collagen fibrils. Gene expression levels on the 30NA substrates were statistically similar to those on 30A substrates.

Previously it has been shown that there is a reduction in the expression of SMA in rabbit corneal fibroblasts grown on aligned collagen fibers compared to cells grown on unaligned collagen substrates.² This difference could be due to the fact that these cells were cultured for a seven day period in media containing FBS, which is an activator of the repair fibroblast phenotype.¹² In our study however, complete media was changed to media without FBS after day one, and cells were harvested on day two for qPCR analysis. The longer exposure time to FBS could possibly amplify the differences in response between cells grown on collagen fibers without and without complete alignment leading to a difference in results between the two studies.

It has long been thought that the presence of monodisperse and aligned fibrils with regular ordered arrangement is required for a transparent cornea.³¹ However, it has been shown that disordered collagen fibrils can still be transparent as long as they do not contain regions of collagen larger than the wavelength of light.^{32,33} These results are in accordance with our data which shows that at the optimal diameter of 30 nm, there is no difference in target gene expression between aligned and nonaligned fibrils.

Conclusion

This study shows that environmental cues such as collagen fibril diameter and alignment may be used to promote the transformation of corneal fibroblasts from the repair phenotype (fibroblasts and myofibroblasts) towards the quiescent phenotype (keratocytes). Our fabrication process allows for self-assembly of molecular collagen into fibrils with control of fibril diameter and alignment. This process is able to deposit highly uniform, transparent collagen scaffolds. Although previous studies have looked at corneal fibroblast alignment, migration⁹ and SMA expression² in response to topography, the expression of corneal crystallins and matrix proteins which play a major role in corneal wound healing has not been investigated. Our study evaluates gene expression levels of the myofibroblast marker SMA, the corneal crystallin, TKT, and matrix proteins such as collagens 1 and 3, fibronectin and biglycan, in response to collagen fibril topography. Furthermore, we have examined the effect of both fibril diameter and alignment on cell morphology and gene expression. Our results show that the expression of SMA is down-regulated, while TKT expression is

increased on all three nanofiber scaffolds (30A, 300A and 30NA) compared to flat collagen coated controls. This suggests that the presence of collagen fibrils attenuates the fibrotic phenotype and promotes expression of the quiescent phenotype, both of which are linked to an increase in cellular transparency in the cornea. Further, matrix synthesis is increased on the 300A substrates, which could be a possible reason for the increased light scattering and loss of transparency observed in scar tissue associated with large diameter fibrils. Our finding that fibril diameter and alignment can affect myofibroblast differentiation, keratocyte regeneration and matrix synthesis suggests that matrix topography may play an important role in corneal development, disease and repair after injury. Future studies that investigate cell response in three-dimensional collagen nanofiber scaffolds and explore the molecular mechanisms involved in the regulation of such responses could benefit the design of tissue engineered corneas and promote our understanding of corneal wound healing processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Table 1

Quantitative Polymerase Chain Reaction Primers

Gene	Primer sequence (5'–3')
COL1	F: CAACCATGCCTCTCAGAA CA R: GCCAGTTTCCTCATCCATGT
COL3	F: GTCCTGATGGTTCCCGTAAA R: TTCAGGATGGCAGAATTCA
Fibronectin	F: TTGATCGCCCTAAAGGACTG R: CATCCTCAGGGCTCGAGTAG
Biglycan	F: TCTCAGAGGCCAAGCTCACT R: TAGCTCGAT TGCCTGGATTT
TKT	F: GACGGGCCCTCCCAGAT, R: CATCACTTGGGTAAAAGACAGTTGA
SMA	F: TGACCGCATGCAGAAGGA R: GGGCGATGATCTTGATCTTCA
GAPDH	F:GCATCGTGGAGGGACTTATGA R: GGGCCATCCACAGTCTTCTG