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Response of Human Corneal Fibroblasts on Silk Film Surface Patterns

Eun Seok Gil, Sang Huyg Park, Jeff Marchant, Fiorenzo Omenetto, and David L. Kaplan*

Department of Biomedical Engineering, Sackler School of Biomedical Science, Tufts University, 4 Colby St., Medford, Massachusetts 02155, USA

Abstract

Transparent, biodegradable, mechanically robust, and surface-patterned silk films were evaluated for the effect of surface morphology on human corneal fibroblast (hCF) cell proliferation, orientation, and ECM deposition and alignment. A series of dimensionally different surface groove patterns were prepared from optically graded glass substrates followed by casting poly(dimethylsiloxane) (PDMS) replica molds. The features on the patterned silk films showed an array of asymmetric triangles and displayed 37–342 nm depths and 445–3 582 nm widths. hCF DNA content on all patterned films were not significantly different from that on flat silk films after 4 d in culture. However, the depth and width of the grooves influenced cell alignment, while the depth differences affected cell orientation; overall, deeper and narrower grooves induced more hCF orientation. Over 14 d in culture, cell layers and actin filament organization demonstrated that confluent hCFs and their cytoskeletal filaments were oriented along the direction of the silk film patterned groove axis. Collagen type V and proteoglycans (decorin and biglycan), important markers of corneal stromal tissue, were highly expressed with alignment. Understanding corneal stromal fibroblast responses to surface features on a protein-based biomaterial applicable in vivo for corneal repair potential suggests options to improve corneal tissue mimics. Further, the approaches provide fundamental biomaterial designs useful for bioengineering oriented tissue layers, an endemic feature in most biological tissue structures that lead to critical tissue functions.

Keywords

cornea; extracellular matrix; fibroblasts; silk; surface patterns

Introduction

Development of tissues from an embryo to an adult is governed by many factors, including chemical and topological cues that impact the final position and orientation of cells and tissue features. In many organ structures, alignment of cells and extracellular matrix (ECM) anisotropy are predominant features that provides critical control of mechanical properties and cell functions. For regenerative medicine, therefore, arrays of different types of cells and ECM should be organized with appropriate orientation and morphological features to match tissue-specific needs. Corneal tissue engineering involves a combination of three major cellular layers with orientation including the outermost epithelium, central stroma, and innermost endothelium.^[1–3]

Corneal replacements are the most commonly performed tissue transplant in the United States, with over 30 000–40 000 performed annually.^[4] However, other options for cornea replacements are in demand because corneal tissue supply cannot satisfy corneal graft demand worldwide. Furthermore, cornea laser surgery further limits the utility of donor cornea. Thus, corneal tissue engineering has gained interest because of the need as well as the achievable avascular and immunological privileged environment. Reconstructing the stroma presents major challenges due to the complexity of well-organized multi-lamella structure with mechanical robustness and optical clarity.^[2,5–8] In contrast to the stromal layers, tissue engineered corneal epithelium^[9] and endothelium^[10] have been successfully developed in animal models due to their relatively simple features. New approaches for engineering corneal equivalents have been proposed by using hydrogel scaffolds,^[5,11,12] porous sponges,^[13] fibers,^[1] and films.^[2,7] Recent efforts have focused on employing corneal fibro-blasts and ECM alignment toward reconstructed stroma.^[1,2,7,11,12] The proper composition and well-aligned microstructure of the stroma determine the biomechanical and optical properties, thus detailed control of cell and ECM deposition become critical to the process.

The response of cells to grooved surfaces has been studied to understand the effect of topological cues on cell orientation.^[14–21] Surface patterns play a role in cell alignment, adhesion, mobility, and proliferation. While responses are cell type-dependent, cells are generally aligned in the direction of the groove on the substrates, known as “contact guidance.”^[22,23] In general, the depth of grooves is been considered as an important factor to induce cell alignment on patterned films, while the width (or ridge) has a minor effect on the cell orientation.^[16,20,24,25] The importance of cell and ECM alignment on patterned surfaces in tissue engineering has been demonstrated with many different types of cells, such as osteoblasts,^[26] cardiac cells,^[27] neurite outgrowth,^[28] muscle cells,^[29] corneal epithelial cells,^[19] fibroblasts,^[2] and vascular cells.^[30] Chemically modified micropatterned collagen films have been studied for their influence of morphological patterns on mesenchymal osteoprogenitor stem cell alignment and differentiation into osteoblasts.^[31] Micropatterned polycaprolactone (PCL) films were also introduced for vascular smooth muscle cell alignment,^[32] and micropatterned silk films have been used to support keratinocyte orientation.^[33] However, fundamental studies are needed with micro- or nanopatterned features using biodegradable biomaterial films where cell behavior and ECM orientation are systematically addressed. Furthermore, it is important to assess both nano- and microscale feature sizes on cellular and ECM outcomes, as this becomes critical

depending on the biomaterial design to be used and on the tissue-specific architectural details targeted, such as corneal tissue in the present contribution.

Grooves have been introduced usually by lithographic macro- or nanofabrication techniques on synthetic substrates such as glass,^[34] metals,^[35] poly(dimethylsiloxane) (PDMS),^[36] and nondegradable synthetic polymers^[16,37] to understand cell behavior on the patterned surfaces, but without consideration of the biological compatibility and direct tissue utility of the substrate.

Silk fibroin has been used as scaffolding material for tissue engineering and regenerative medicine applications due to controllable degradability, tunable, and robust mechanical properties and nonimmunogenic response upon in vivo implantation.^[38–43] Optically transparent silk fibroin films can also be fabricated with controllable elasticity and degradability,^[39] porosity,^[2] and micropatterned surfaces.^[2,33] Topographic patterns on silk film surfaces can be introduced by soft lithographic or micro-machining techniques.^[44,45] In our previous study, we showed the potential to bioengineer corneal stroma using patterned silk films which can direct cellular morphology and ECM orientation.^[2]

In the present study, we investigated the influence of different surface pattern features on human corneal fibroblast (hCF) response. Optically transparent patterned silk films were used for the study with clinically applicable thickness (2 μm) toward corneal tissue engineering.

Experimental Part

Preparation of Silk Solution

Silk solution was prepared from *Bombyx mori* silkworm cocoons according to the procedures described in our previous studies.^[39,43] Cocoons of *B. mori* silkworm silk were supplied by Tajima Shoji Co. (Yokohama, Japan). Briefly, the cocoons were degummed in a boiled 0.02 M Na_2CO_3 (Sigma–Aldrich, St. Louis, MO) solution for 30 min. The fibroin extract was then rinsed three times in Milli-Q water, dissolved in a 9.3 M LiBr solution yielding a 20% w/v solution, and subsequently dialyzed (MWCO 3 500) against distilled water for 2 d to obtain silk fibroin aqueous solution (ca. 8% w/v).

Preparation of Poly(dimethylsiloxane) (PDMS) Substrates

Patterned PDMS (GE Plastics, Pittsfield, MA) substrates 1–2.0 mm thickness were prepared by casting on a series of reflective diffraction gratings (Edmund Optics, Inc., Barrington, NJ). The features of the diffraction gratings are listed in Table 1. The PDMS substrates were punched into round disks with 14 mm or 11 diameters. The PDMS substrates were washed in a 70% ethanol and then thoroughly rinsed in DI water before casting silk solutions on the substrates to generate the patterned films.

Preparation of Silk Films

For film preparation, a 100 or 62 μL of 1% silk solution was cast on the prepared PDMS substrates, 14 mm or 11 mm in diameter, to generate films with a thickness of around 2 μm .^[46] The as-cast silk films were water-annealed in a water-filled desiccator at 24 mm Hg vacuum for 5 h. The silk films were immersed in a water bath and removed from the PDMS substrates, and then placed into 24-well plates with the grooved surface facing up. The films were sterilized with 70% EtOH, washed three times in PBS (pH 7.4), and dried. The wells were then filled with 0.5 mL media overnight before cell seeding.

Surface Characterization

Surface structures of patterned or flat silk films were characterized using scanning electron microscopy (SEM) and atomic force microscopy (AFM). For SEM images, the fractured-sections of the patterned films were obtained in liquid nitrogen using a razor blade. The samples were sputter coated with Pt/Pd. The morphology was examined with a Field Emission SEM (FESEM) (Zeiss Ultra55 or Supra55VP, Carl Zeiss AG, Oberkochen, Germany) operating at 6 kV. The features of the patterned silk films were quantitatively evaluated using a Digital Instrument Dimension 3100 AFM (Veeco Instruments, Inc., Woodbury, NY) in tapping mode. The scan direction was vertically adjusted against groove direction. The scan rate was $10 \mu\text{m} \cdot \text{s}^{-1}$ and the scan distance was $10 \mu\text{m}$ with aspect ratio of 3. Surface depth, width, and RMS roughness were measured using Nanoscope imaging software (Veeco) and averaged for 10 random fields per substrate.

Transfected Human Cell Culture

P7 immortalized human corneal keratocytes (hCFs) were kindly provided by May Griffith (University of Ottawa Eye Institute). The human cell lines were isolated from donor cornea and immortalized by infection with an amphotropic recombinant retrovirus containing HPV16 genes E6 and E7 and with mammalian expression vectors containing genes encoding SV40 large T antigen, pSV3neo, and adenovirus E1A 12S, as described previously.^[3] The cells were cultured in DMEM medium containing 10% FBS, 1% interferon-transferrin-selenium (ITS), and 1% PSF (Gibco, Pascagoula, MS). The cultures were harvested with 0.05% trypsin with $0.4 \times 10^{-3} \text{ M}$ EDTA.

DNA Content Assay

The effect of the different grooved surface features on hCF viability was evaluated using the Pico Green assay (Invitrogen, Inc., Grand Island, NY). hCFs were seeded on silk films at a density of $5\,000 \text{ cells cm}^{-2}$ and cultured for 4 d before measuring DNA content. For the Pico Green assay, samples were washed twice with PBS and subsequently incubated in 0.5 mL of 0.1% Triton-X 100 in $1 \times \text{TE}$ buffer for 48 h. The sample supernatant was spun down by centrifugation for 5 min at 10 000 rpm. Subsequently, 25 μL of supernatant and 75 μL of $1 \times \text{TE}$ buffer were placed into 96-well plates and 100 μL of a 1:200 dilution of Quant-iT PicoGreen (Invitrogen, Inc.) reagent was added to each well and read using a fluorimeter with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Cell Alignment Analysis

hCFs were seeded on the silk films at a density of $5\,000 \text{ cells} \cdot \text{cm}^{-2}$ and phase contrast images examined on the second day with a Leica/Leitz DM RBE Microscope (Leica, Mannheim, Germany) or Zeiss Axiovert 40 CFL Microscope (Carl Zeiss AG). The orientation of hCFs was analyzed with ImagePro 6 software. The orientation angle was determined by calculating the angle difference between the longest direction within the cell borders and the silk film grooves. Over 2 d in culture, the angle difference between the longest axis of the cell boundary and the groove direction was measured from all existing cells shown in $5 \times$ phase contrast images. The mean orientation angles of hCFs grown on the flat and all other patterned films were calculated from three individual $5 \mu\text{m}$ phase contrast images. Also, the populations of cells whose angles were in each 10° region from the groove axis were plotted from 0 to 90° .

Cytoskeleton and ECM Alignment

hCFs were seeded at a density of $20\,000 \text{ cells} \cdot \text{cm}^2$ on the silk films and cultured for 2 weeks before taking images. Cell culture medium was gently removed and the silk films in culture plates were gently washed twice with PBS (pH 7.4). Subsequently, the samples were

fixed for 10 min at room temperature using 4% paraformaldehyde solution. The 4% paraformaldehyde was removed with three subsequent PBS washings. Then the cells were permeabilized with PBS (pH 7.4) containing 0.2% Triton X-100 for 10 min, and blocked with PBS (pH 7.4) containing 1% BSA for 30 min. Actin filaments were stained using Texas Red-X phalloidain stain (Invitrogen, Inc.), which was diluted using 10 μL of methanolic stock reagent and 400 μL of PBS for each sample. After diluting, 410 μL was placed onto each sample for 30 min with two subsequent PBS rinses. Primary antibodies for collagen type V (Invitrogen, Inc.), decorin (Abcam, Inc., Cambridge, MA), and biglycan (Abcam, Inc.) were diluted from their respective stock solutions to 5–10 $\mu\text{g mL}^{-1}$ concentrations in PBS. 250 μL of antibody solution was placed onto each sample in 24-well plates and incubated at 4 °C for overnight. The samples were then washed three times with PBS and stained using Alexafluor 488 (Invitrogen, Inc.) as secondary antibody, in which a 10 $\mu\text{g mL}^{-1}$ dilution was prepared. A 400 μL aliquot of secondary antibody solution was added to each sample for 30 min with two subsequent PBS rinses. All samples were mounted onto glass slides using Prolong anti-fading mounting media (Invitrogen, Inc.). Confocal microscopy was carried out to examine cytoskeleton and ECM alignment on the patterned silk films. The middle z-section images of cells were taken by a Leica TCS SP2 AOBS confocal microscopy (Leica) equipped with 488 nm argon and 543 nm He/Ne lasers. Phalloidain staining excitation was at 595 nm and collected emission between 605 and 650 nm and Alexafluor 488 secondary antibody excitation was at 488 nm and collected emission between 500 and 550 nm.

Statistical Methods

Results were statistically analyzed using one-way analysis of variance (ANOVA). A statistically significant difference was reported if $p < 0.05$ or less. Data are reported as the mean $\pm$ standard deviation (SD) from at least three separate experiments.

Results

Characterization of Patterned Films

The fabrication procedure for patterned silk films with controllable film thickness is shown in Figure 1.^[46] We used a series of reflective diffraction grating glass substrates that are commercially available as patterning molds. The SEM images display top and side views of representative patterned silk films whose thicknesses are 2 μm (Figure 1b–e). The grooves featured an asymmetric triangle array with well-controlled features and the dimensions and roughness are listed in Table 1. The surface grooves on the silk films ranged from 445 to 3584 nm in width and from 37 to 342 nm in depth, respectively. AFM images showed an RMS roughness of 3.2 ± 0.5 nm on flat film surfaces, and 11.6 ± 1.6 – 120.5 ± 1.1 nm on the patterned films. Overall, RMS roughness increased as groove depth increased, while the change in groove width did not have an effect on RMS roughness.

hCF Alignment and Proliferation on Patterned Films

After hCFs were cultured on flat and grooved film surfaces for 2 d, image analysis and subsequent statistical analysis were carried out from three $5\times$ phase contrast images (Figure 3a–c). Cell angle distributions were determined from the groove axis (Figure 4 and 5), and the aligned hCFs percentages within 10 or 20° from the groove axis are listed in Table 2. From the phase contrast images of hCFs on the patterned silk surfaces over 2 d post-seeding, cell alignment was evident in representative patterned samples F and H (Figure 3b and c), while the hCFs were randomly oriented on flat silk surfaces (Figure 3a). Figure 4 displays histograms of cell angle distribution from groove axis for every 10° from 0 to 90°. From these plots, aligned cell populations were calculated within 10 or 20° (Table 2). Groove depth affected cellular orientation; the aligned cell population within 10° (or 20°) on sample

F (187 ± 9.1 nm in depth) and H (342 ± 18.0 nm in depth) showed 53% (77%) and 73% (89%), respectively, whereas those on the samples that had a shallower depth than 100 nm exhibited lower values, ranging from 20 to 28% (35–48%). We also observed a significant depth effect on cellular alignment in the mean cellular orientation angles from the groove axis (Figure 5a). hCFs cultured on the flat silk films showed a mean cellular orientation angle at around 45° , while hCFs cultured on the various patterned films exhibited significantly reduced mean cellular orientation angles (9 – 36°) compared to the flat films. In addition, the mean cellular orientation angles significantly decreased when feature depth increased with the same width. The width of grooves also slightly affected cell alignment (Figure 5b). A lower mean cellular orientation angle was observed with narrower width with sample C (width: 866 nm, angle: 24.2 ± 1.78), E (width: 1796, angle: 30.0 ± 3.08), and G (width: 3499, angle: $34.5 \pm 0.5^\circ$), whose groove depths were almost the same (65–85 nm). Overall, deeper and narrower groove features on the silk film surfaces resulted in higher hCF orientation.

At day 14, when hCFs on all silk films were confluent, samples F and H exhibited confluent cell coverage aligned along the groove axis direction over the entire surface area (Figure 3e and f). Although the other patterned samples showed oriented cell bodies on most of surface area, they also showed some failures of cell body orientation along the groove axis in partial surface areas (images not shown). In contrast, the flat surface showed confluent random growth (Figure 3d).

DNA content of hCFs grown on both flat and all patterned silk surfaces was measured after 4 d in culture (Figure 6). The data indicated no significant difference in DNA content between flat and all patterned silk films or all grooved silk films.

Cytoskeleton Alignment and Extracellular Matrix (ECM) Orientation on Patterned Silk Film

Over the period of 14 d in culture, confocal images of hCF actin stained with phalloidin showed actin filament alignment along the patterned groove axis in all patterned films, including sample A, which showed only $21.2 \pm 1.8\%$ of hCFs aligned within 108 along the groove axis after 4 d in culture (Figure 7, upper images). Except for samples F and H, the other patterned samples with groove depths were lower than 100 nm exhibited some failures of actin alignment along the patterned groove axis in partial area (images not shown) as observed in confluent cell coverage. In contrast, actin filaments were randomly formed on flat silk film surfaces. These results agree with cell body orientation on the patterned and flat silk film surfaces when hCFs were confluent (after 14 d in culture) as shown in Figure 3d–f.

Immunocytochemistry confocal images were obtained to examine the expression and orientation of collagen type V and representative proteoglycans (decorin and biglycan), as corneal stroma markers, on both flat and patterned silk films (Figure 7, lower images). High expression and alignment of these components on the patterned silk films were displayed along the groove axis. In contrast, the collagen type V and proteoglycans were randomly aligned on the flat silk film in spite of their high level of production. The expression of collagen type V and proteoglycans suggests that the hCFs retained their differentiated phenotype on the silk films.

Discussion

The present study has demonstrated a biomimetic approach to replicate corneal stromal tissue architecture using optically transparent and surface patterned silk films. The aim of the study was to understand the morphological response of hCFs and their ECM production and alignment to topologically different groove patterns on thin silk films. Topological cues, termed “contact guidance,”^[22] can induce biomimetic tissue structures in both cells and ECM. In many tissue engineering applications, it is important to reconstruct oriented

features of cells and ECM with scaffolding biomaterials,^[2,19,27–31] particularly if the helicoidal nature of most tissue microstructures is to be adequately recapitulated for appropriate structure and function. The morphological behavior of different types of cells on the micro and nanogrooved substrates has been addressed in many studies.^[16,20,24,25] These studies include an effort to find thresholds of groove dimension at which effective cell alignment occurs.^[20] However, these experimental conditions utilize nondegradable substrates (e.g., glass, metal, or synthetic polymers) and may not be relevant in the case of tissue engineering applications that require implantable degradability for full tissue regeneration.

The use of surface patterning may be coupled with appropriate biopolymers to suggest controlled material–cell interactions, thus offering a highly tailored biomaterial approach to implantable medical devices. However, the relationship between topographic surface patterns of biopolymer materials and cell behavior is not understood. Therefore, this study offers insight into biomaterial fabrication strategies for tissue engineered corneal constructs but also for many other devices that could be envisioned related to lamellar structures with appropriate control of alignment and orientation of cells and ECM.

To produce different surface groove patterns on the silk films, ruled diffraction gratings designed for optical diffraction were used as replica substrates. The manufacturer describes that fine ruling was performed by precise, interferometrically controlled ruling engines, forming a sawtooth-shaped groove profile. Through the fabrication method shown in Figure 1, eight different grooved features on the films were obtained and compared with unpatterned surfaces. The groove structures had different depths and widths to allow us to investigate these effects on hCF responses. To apply grooves on a free standing silk film, the groove depth should be sufficiently shallower than film thickness, therefore, the nanoscale range of the groove depths matched our effort to reconstruct corneal equilibrants using free standing 2 μm thick silk films. The surface features on these thin silk films formed an asymmetric triangle array (asymmetric V-shape) with steep and gentle slopes in both sides. The groove shape seems unique compared to the rectangular or symmetric grooves in other studies.^[14] One steep slope in each triangle could function to guide cells, while the more gradual feature could support cell adhesion on its major surface area. The combination of steep and gentle angles should provide appropriate contact guidance for cell and ECM without sacrificing cell adhesion.

The DNA content of hCFs grown on these surfaces over 4 d in culture demonstrated that the presence of the surface grooves or the dimension of the groove features on the surface did not significantly affect cell proliferation. These results were also found in studies with rat dermal fibroblasts^[47] and human skin fibroblasts^[48] grown on silicon wafer and PDMS, respectively. On all grooved silk film surfaces, cell orientation was observed, while the degree of orientation depended on groove dimension. Overall, deeper and narrower groove structures produced more hCF orientation. However, the depth of grooves was more important for cell alignment. Other studies have also reported that the depth of grooves was more important for cell alignment than width.^[14–17] It was reported that human dermal fibroblasts aligned on the patterned substrates of fused quartz, with groove dimensions 110–3 800 nm in depth and 1–100 μm in width, while cell alignment was more predominant with deeper and narrower grooves.^[23] No cell alignment was observed when the depth of the grooves was 27 and 44 nm. The dimensional threshold of nanogrooves was studied, resulting in fibroblast cell orientation with over 35 nm in depth and over 100 nm in width on the substrates of silicon wafer.^[20] The present results confirmed these other studies, regardless of different groove shape and differences in substrate material characteristics. Therefore, cell alignment on the grooved silk films seemed to follow similar contact guidance as on other substrates. For degradable substrates, micropatterned collagen films,

with dimensions of groove depth, width, and ridge 12, 2, and 27 μm , respectively, induced hMSC orientation.^[31] Micropatterned PCL films with 5 μm depth, 48 μm grooves, and 12 μm spacing, guided vascular smooth muscle cell alignment.^[32] Micropatterned silk fibroin films with groove spacing of 10 and 2 μm depth supported keratinocyte proliferation, differentiation, and alignment.^[33] However, these groove features were large compared to the reported dimensional thresholds where such morphologies can affect cell alignment in order to more fully understand the influence of dimensional patterns on cells. Among the groove structures studied on the silk films, hCFs grown with 180 nm (sample F) and 340 nm (sample H) depths showed the most flawless cell body and cytoskeleton orientation over the entire film area along the groove axis after confluent growth. Therefore, samples F and H may be the most useful morphologies for corneal tissue reconstruction in future studies.

Despite many studies in cell behavior on grooved substrates, the ECM generated on these features and the alignment morphology has not been extensively demonstrated. Stem cell-derived osteoblasts showed cell body and cytoskeletal alignment, and, mineralized matrix alignment, on polystyrene nanogrooved substrates (300 nm pitch, 60–70 nm depth).^[26] In our previous report, on patterned silk films (note: sample F was used), rabbit corneal fibroblasts displayed anisotropic orientation not only in cell and actin fibrils, but the cells also expressed collagen type V.^[2] In this study, we further studied hCF and actin alignment, and ECM expression and orientation. Collagen V is highly expressed as characteristic of the corneal fibroblast phenotype and functions in regulating collagen fibril diameter.^[49,50] Corneal fibroblasts can differentiate into a myofibroblast phenotype upon injury, where they do not highly express collagen type V during scar formation.^[51] Also, decorin and biglycan are class I small leucine-rich proteoglycans (SLRPs) that play a role in the regulation of collagen fibril and matrix assembly in the ECM of connective tissue including corneal stroma. Decorin is expressed in all developmental stages, while biglycan is highly expressed only at an early stage, decreasing during development, and being present at very low levels in the mature cornea.^[52] Collagen type V and proteoglycan (decorin and biglycan) expression and alignment were observed along the groove direction on the patterned silk films. These results demonstrated that the hCFs retained their differentiated phenotype while cultured on the silk films. In addition, both decorin and biglycan were highly expressed on both flat and patterned films, indicating that the development of corneal stroma seems to be in an early stage of formation over a period of 14 d in culture, just after hCFs were confluent.

Conclusion

The effect of surface groove features of thin silk films was evaluated with hCFs, including cell proliferation, cell orientation, and ECM alignment. Unique surface groove patterns were introduced on the silk film surfaces using optically graded glass substrates followed by casting PDMS replica molds. The patterns on the thin silk films (2 μm thickness) was modulated to have asymmetric V shape grooves featuring from 37 to 342 nm depths and from 445 to 3 580 nm widths. Both depth and width of grooves affected cell alignment, while the depth difference had a greater impact on cell orientation. Overall, deeper and narrower groove structures on the silk film surfaces produced more hCF orientation. The DNA content was not significantly changed with different surface structures after 4 d in culture. Over a period of 14 d in culture, hCFs expressed collagen type V and proteoglycans as markers for corneal stroma. Cell bodies, actin filaments and expressed ECMs were confluent and highly aligned on the patterned silk films along the groove directions. The aligned hCFs and ECMs with proper cornea stroma phenotype on the patterned silk films imply that these silk film substrates can be used in mono- or multilayer systems as an approach to reconstruct corneal tissues. Moreover, this study suggests that patterned silk films could be envisioned for engineering other oriented tissue structures including neural,

muscular, and cardiac tissue beyond just corneal tissue engineering, due to the dominating importance of helicoidal structures as a core feature in tissue structure and function.

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Figure 1. Fabrication of patterned silk films. Schematic illustration of patterned silk film production. (a) SEM micrographs of silk films with 2 μm thickness: sample C (b), sample F (c), and sample H (d) (see Table 1). Groove features on silk films (e), where the width and depth range 445–3 582 and 37–342 nm, respectively.

Figure 2. AFM images of 2D and 3D topographies and height profiles of flat and grooved silk film surfaces: flat (a), samples A, F, and H (b, c, and d, respectively).

Figure 3. Phase contrast optical micrographs of hCFs grown on flat (a, d) and grooved silk films; sample F (b, e) and H (c, f), over 2 d (a–c) and 2 weeks in culture (d–f).

Figure 4.

Histograms for angle differences between cells and groove axis. Percent of cells plotted every 10° from 0 to 90 on the films: (a) samples A, (b) B, (c) C, (d) D, (e) E, (f) F, (g) G, (h) F, and flat film (i) after 2 d in culture. The orientation angle was determined by calculating the angle difference between the longest direction within the cell borders and the grooves. The x-axis represents cell angles from the groove direction, and the y-axis is cell population shown as percent. Angles between the longest axis of the cell boundary and the groove direction are measured from all existing cells shown in three individual 5× phase contrast images ($N=168-291$).

Figure 5.

Mean orientation angles of hCFs grown on the flat and patterned silk films. Data were obtained from three individual $5\times$ phase contrast images after 2 d in culture. The orientation angle was determined by calculating the angle difference between the longest direction within the cell borders and the grooves. The mean orientation angle was defined by the $\text{mean} \pm \text{SD}$ of three orientation angles averaged from three individual $5\times$ images. Plots depict the effects of groove depth (a) and width (b) on the orientation angle ($N=3$, Cell population in each image varied from 40 to 123. Bars represent SD).

Figure 6. DNA content for hCFs grown on flat and patterned silk film substrates after 4 d in culture [no significant differences were observed among all data ($p < 0.05$) $N=4$. Bars represent SD].

Figure 7.

Confocal images of hCFs after 14 d in culture on flat and patterned silk films. Alphabet on the left top in each figure represents the name of samples. Red (upper images) indicates stained actin filaments, while green (lower images) represents immunostained collagen V (COL), decorin (DCR), and biglycan (BGN).

Table 1

Dimension and roughness of grooves on silk film surfaces.

Samples	Depth (nm±SD)	Width (nm±SD)	Roughness (nm±SD)
Flat	—	—	3.2±0.5
A	39±5.3	445±33	11.6±1.6
B	37±3.5	865±27	13.0±0.3
C	65±4.1	866±26	23.3±0.2
D	64±6.1	1796±173	19.5±1.3
E	86±5.9	1792±103	30.2±1.6
F	187±9.1	1832±68	64.3±1.1
G	85±8.3	3499±117	25.0±2.6
H	342±18.0	3584±108	120.5±1.1

Table 2

Cellular orientation on flat and grooved silk films after 2 d in culture.

Samples	Aligned hCF cells within 10° from groove (%±SD)	Aligned hCF cells within 20° from groove (%±SD)
Flat	11.8±5.2 ^{a)}	22.3±5.7 ^{a)}
A	21.2±1.8 ^{b)}	37.0±6.9 ^{b)}
B	25.8±5.8 ^{b)}	43.2±5.2 ^{b)}
C	27.7±2.1 ^{b)}	47.4±4.5 ^{b)}
D	20.0±5.3 ^{b)}	34.7±4.0 ^{b)}
E	21.4±4.8 ^{b)}	47.5±2.4 ^{b)}
F	52.7±8.7 ^{b)}	76.9±6.6 ^{b)}
G	23.5±5.2 ^{b)}	36.4±7.3 ^{b)}
H	72.6±3.1 ^{b)}	88.5±6.9 ^{b)}

^{a)} Groove angle of flat film was considered as vertical direction in the image;^{b)} $p < 0.05$ or less compared to flat sample.