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Surface Topography and Mechanical Strain Promote Keratocyte Phenotype and Extracellular Matrix Formation in a Biomimetic 3D Corneal Model

Wei Zhang, Jialin Chen, Ludvig J. Backman, Adam D. Malm, and Patrik Danielson*

The optimal functionality of the native corneal stroma is mainly dependent on the well-ordered arrangement of extracellular matrix (ECM) and the pressurized structure. In order to develop an in vitro corneal model, it is crucial to mimic the in vivo microenvironment of the cornea. In this study, the influence of surface topography and mechanical strain on keratocyte phenotype and ECM formation within a biomimetic 3D corneal model is studied. By modifying the surface topography of materials, it is found that patterned silk fibroin film with 600 grooves mm\(^{-1}\) optimally supports cell alignment and ECM arrangement. Furthermore, treatment with 3% dome-shaped mechanical strain, which resembles the shape and mechanics of native cornea, significantly enhances the expression of keratocyte markers as compared to flat-shaped strain. Accordingly, a biomimetic 3D corneal model, in the form of a collagen-modified, silk fibroin-patterned construct subjected to 3% dome-shaped strain, is created. Compared to traditional 2D cultures, it supports a significantly higher expression of keratocyte and ECM markers, and in conclusion better maintains keratocyte phenotype, alignment, and fusiform cell shape. Therefore, the novel biomimetic 3D corneal model developed in this study serves as a useful in vitro 3D culture model to improve current 2D cultures for corneal studies.

1. Introduction

The cornea is the outermost transparent part of the eye, pivotal for functioning vision since its main role is to refract light and protect deeper structures.\(^1\) It consists of five different layers:

- The outermost epithelium, Bowman’s layer, the stroma, Descemet’s membrane, and the innermost endothelium.\(^2\) The stroma, which constitutes up to 90% of the corneal thickness, is composed of well-organized collagen fibrils and quiescent stromal cells called keratocytes.\(^3,4\) The structure of the stroma is important for the transparency and consequently the vision. The properties of the stroma are based on the combination of the orthogonal lamellar arrangement of aligned collagen fibrils (mainly collagen I and V)\(^5\) and the spacing of these fibrils and the regulation of collagen diameter achieved by proteoglycans (such as lumican and keratocan).\(^6,7\)

   It has been shown that keratocytes during in vitro cell culture conditions easily differentiate, as shown by reduced expression of the typical keratocyte markers, including keratocan, CD34, and ALDH3A1.\(^13,14\) However, most of the in vitro corneal studies are conducted on 2D monolayers.\(^15-17\) To overcome the limitations of 2D monolayer cell cultures, several 3D corneal in vitro models have been developed using tissue-engineered strategies and a number of biomaterials, such as collagen, silk, chitosan, and other synthetic polymers.\(^14,18\) Among the various materials, collagen is the most commonly used, as it is the main constituent of native cornea, and collagen-based corneal 3D models have shown promising results as substrates for the culture of corneal cells.\(^19-21\) However, few of the 3D corneal in vitro models can replicate the in vivo microenvironment of the native cornea, due to the complexity of the corneal structure and the uniqueness of the mechanical environment created by the corneal shape and the intraocular pressure. To engineer a functional corneal in vitro model, it is therefore imperative to better understand the structure of the cornea and the effects of a variety of signals seen in vivo, such as mechanical stimuli.

The lamellae-like tissue is one of the most critical features of the corneal stroma.\(^6,7\) To reproduce this feature, materials with topographical cues are generated by surface-patterning.\(^22-25\) Recently, silk fibroin has been widely used in corneal tissue engineering strategies due to its good biocompatibility, excellent mechanical strength, controllable biodegradability, and noninflammatory properties.\(^22,26-29\) Kaplan and collaborators reported successful fabrication of a surface patterned, mechanically robust, and transparent silk fibroin film, which could support corneal fibroblast proliferation, alignment, and corneal

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extracellular matrix (ECM) expression. It provided a feasible way to mimic the microstructure and arrangement of collagen fibers of native cornea, but the silk material has seldom been evaluated in a 3D corneal model that closely mimics corneal microenvironment.

It is well-known that mechanical strain influences the properties and behavior of cells in vivo. The cornea is a pressurized structure resulting in a dome-shaped strain of the cells in the stroma, varying from small strains up to 7% depending on the location in the cornea. Most commonly, the strain is within the range of 0%–3%. The fact that the native cornea is a pressurized structure suggests that mechanical strain may be important to maintain the microenvironment of this tissue and the phenotype of the keratocytes. In other cell types, it has been shown that mechanical stimuli in vitro is effective in promoting the characteristics of specialized cells, not least tendon cells (tenocytes). Whether keratocytes that are exposed to mechanical strain in vitro, in conditions mimicking those of the native cornea, show properties more similar to those of healthy keratocytes in vivo, is not yet known.

Hence, this study aimed to develop a biomimetic 3D corneal in vitro model that combines the topographical and mechanical cues similar to those of the native cornea. The aim was furthermore to study whether this 3D corneal in vitro model can superimpose keratocyte phenotype and ECM formation as compared to the traditional 2D cell culture model. To meet these objectives, experiments were arranged as follows: (1) to evaluate the effect of surface topography by fabricating unpatterned/patterned (300 or 600 grooves mm–1) silk fibroin films; (2) to evaluate the effect of mechanical strain by applying strain of different strength (0%–6%) or different shapes (dome/flat) using the Flexcell Tension System; and, based on the above results, (3) to evaluate the synergistic effects of surface topography and mechanical strain.

2. Results and Discussion

2.1. Fabrication and Optimization of the Silk Fibroin Films

The native corneal stroma is a multilaminar structure consisting of a stack of orthogonal lamellae of aligned collagen fibrils. In the present study, this topographical feature was mimicked by film deposition technique with silk fibroin biomaterial as previously reported. To prevent the silk fibroin film from dissolving in water, water-annealing treatment was adopted, which induces the β-sheet formation and generates physical crosslinks in the crystals. In contrast to the commonly-used water-annealing protocols (1 d at room temperature), in our study the water-annealing time was further optimized as we found that the different water-annealing time had marked difference in impact on the film stability and cell proliferation. Silk fibroin films with different water-annealing time ranging from 2 to 24 h were tested. All the films showed comparative and acceptable transparency when immersed in Phosphate-buffered saline (PBS) for 1 d (Figure 1A), whereas after 7 d in PBS, the transparency of films of 2 h water-annealing processing significantly decreased as compared to those of 4/6/8/16/24 h processing (Figure 1B, P < 0.001 to all other groups). The decrease of β-sheet content led to a less compacted structure and faster degradation as previously reported, and therefore the films of 2 h water-annealing became opaque and uneven which indicated instability of the films (Figure 1C).

The keratocyte attachment and proliferation on the films were also evaluated. The films of 2/4/6 h water-annealing supported more cell attachment to the films after 1 d, and faster proliferation after 4 and 7 d, than those of 8/16/24 h water-annealing, as determined under microscopic observation (Figure 1D). Additionally, the MTS cell proliferation assay at day 7 indicated that the films of 2/4 h water-annealing were more favorable to keratocyte proliferation as compared to the films of 8/16/24 h water-annealing (Figure 1E, 2/4 h vs 8 h: P < 0.01; 2 h vs 16 h: P < 0.05; 4 h vs 16 h: P < 0.01; 2/4 h vs 24 h: P < 0.05).

In light of these data, we fabricated the silk fibroin films using 4 h water-annealing for the following experiments.

2.2. The Topographical Effects on Keratocyte Behavior

To replicate the structure of native cornea, patterned silk fibroin films with either 300 or 600 grooves mm–1 spacing were fabricated. Both the patterned silk fibroin films (regardless of 300 or 600 grooves mm–1) and the unpatterned silk fibroin films were optically clear, very thin, and displayed smooth surfaces under macroscopic observation (Figure 2A). The light transmission of all silk fibroin films exceeded that of the native human cornea, i.e., >87%, regardless of the surface topography (Figure 2B). Scanning Electron Microscope (SEM) images revealed the surface morphology and the cell response on different types of films (Figure 2C). The flat surface of the unpatterned silk film showed a randomly oriented cell growth. The patterned silk fibroin films displayed evident grooves on the surface, which were about 3 and 1.5 µm wide for the 300 and 600 grooves mm–1 films, respectively. Cell alignment along the grooves, as well as more elongated cell morphology than on unpatterned films, were observed on the patterned silk films, both of which were more evident on the 600 grooves mm–1 film compared with the 300 grooves mm–1 film. Similarly, F-actin staining exhibited more aligned and elongated cell morphologies on the 600 grooves mm–1 patterned surfaces as compared to those on the unpatterned or 300 grooves mm–1 films (Figure 2D). Quantitative evaluation of cell alignment further showed that the 600 grooves mm–1 patterned silk fibroin films had a mean cellular orientation angle of 5.94° in relation to the axis of the grooves, indicating that cells were well-aligned, which was significantly lower than that of the unpatterned (58.90°, P < 0.001) or 300 grooves mm–1 (29.68°, P < 0.001) films (Figure 2E). As a conclusion of these results, the 600 grooves mm–1 silk film, with the 1.5 µm wide grooves, was selected as the optimal patterned film to study the topographical effects (hereafter referred to as “patterned”). This is consistent with previous studies using 600 grooves mm–1 silk films for the culture of corneal cells to mimic the lamellar structure of cornea. Collagen fibrils in the native corneal stroma have a diameter of 25–35 nm. This diameter is so small that it is difficult to achieve by the film deposition technique. Nevertheless, the 1.5 µm wide grooves on the silk film used here, were still shown to favorably guide the alignment of cells and ECM synthesis, and thus mimic...
the lamellae-like structure of native cornea, which is of great importance for corneal transparency. The MTS assay revealed comparable cell proliferation on the unpatterned and patterned silk fibroin films on days 1, 4, and 7 (Figure S1A in the Supporting Information, \( P > 0.999 \) for day 1 and 4, \( P = 0.062 \) for day 7). In addition, there was no significant difference between the unpatterned and patterned groups on the gene transcript levels of the keratocyte markers (lumican \( P = 0.657 \) and keratocan \( P = 0.826 \)), or matrix formation markers (collagen I \( P = 0.524 \) and collagen V \( P = 0.712 \)), as evidenced by real-time Polymerase Chain Reaction (PCR) analysis (Figure S1B, Supporting Information).

The immunofluorescent staining showed that both collagen I and collagen V, the representative collagen types of corneal stroma, were well-aligned along with the groove axis on the patterned silk surfaces, but randomly oriented on the unpatterned silk surfaces, which was consistent with that of the F-actin alignment (Figure 2F). These data suggest that keratocytes are able to synthesize ECM components resembling the highly organized structure of native corneal stroma on the patterned silk fibroin films.

Consistent with previous studies,\(^{[22,27]}\) our pattern-structured silk films provided a transparent appearance and not only supported cell attachment and proliferation, but also successfully guided the alignment of keratocytes and collagen fibrils in the direction of the grooves, which resembles the highly organized structure of the native cornea. However, in contrast to our results, previous studies have shown that the surface topography of materials may risk altering the gene expression of keratocyte markers and ECM synthesis.\(^{[27,40–42]}\) The discrepancies to our results in this regard may owe to the different materials used in the previous studies (collagen, poly-caprolactone, etc.) and the different surface topography applied in those studies (pattern diameters, pore structure etc.), suggesting that the patterned silk fibroin film used here can be further optimized to promote keratocyte phenotype and ECM production.

2.3. The Mechanical Effects on Keratocyte Behavior

As the native cornea applies a dome-shaped strain on keratocytes in vivo, we first studied if an in vitro environment that mimics the dome-shaped strain of the cornea better preserves the keratocyte phenotype and has a matrix formation profile more like the one seen in vivo, than a model of flat-shaped...
strain or no strain. The loading posts of 25 mm in diameter, both the dome-shaped (Figure 3A) and the flat-shaped (Figure 3B), were custom-made. Accordingly, the Flexcell system provides a dome-shaped or flat-shaped mechanical strain to the cells cultured on the different loading posts. Since the corneal transparency is to a large extent attributed

Figure 2. Evaluation of the unpatterned and patterned silk fibroin films. A) The photos of silk fibroin films showed that they were visually clear enough to see the letters below. B) The measurement of light transmittance of the films following 1 d immersing in PBS. C) SEM micrographs of the unpatterned, 300 and 600 grooves mm⁻¹ patterned silk fibroin films (left column; 5000×, scale bars = 10 µm), and SEM micrographs showing the keratocytes cultured on them at day 3 (right column; 1000×, scale bars = 20 µm), respectively. D) F-actin staining of the keratocytes seeded on the unpatterned, 300 and 600 grooves mm⁻¹ patterned silk fibroin films at day 3, respectively. The arrows indicated the groove direction of the patterned films. Scale bars = 100 µm. E) Mean cellular orientation angles of keratocytes grown on the unpatterned, 300 and 600 grooves mm⁻¹ patterned silk fibroin films. Results are shown as mean ± SEM. *** P < 0.001. F) Immunofluorescent staining of ECM deposition on unpatterned and patterned (600 grooves mm⁻¹) silk fibroin films at day 7. Red indicates collagen I or V (first and fourth column, respectively), green indicates F-actin and blue indicates stained nuclei using DAPI. The arrows indicate the groove direction of the patterned films. Scale bar = 100 µm.
to the parallel alignment of lamellae (mainly collagen I and V)\textsuperscript{[5–8]} and the uniform spacing between them by proteoglycans (such as lumican and keratocan)\textsuperscript{[9–12]} the gene and protein expression levels of lumican, keratocan, collagen I, and collagen V were compared among the unstrained, 3% dome-shaped strained and 3% flat-shaped strained groups after 3 d. The mRNA expression of the keratocyte marker lumican was significantly enhanced in the dome-shaped strained group as compared to both the flat-shaped strained and the unstrained groups (Figure 3C, $P < 0.01$ to unstrained, $P < 0.001$ to flat-shaped strain). Additionally, the dome-shaped strain also significantly increased the ECM markers collagen I (Figure 3E, $P < 0.01$ to unstrained and flat-shaped strain) and collagen V (Figure 3F, $P < 0.01$ to unstrained, $P < 0.05$ to flat-shaped strain), as compared to both the flat-shaped strained and the unstrained groups. Keratocan gene expression was significantly increased in the dome-shaped strained group as compared to the flat-shaped strained group ($P < 0.01$) but not to the unstrained group ($P = 0.078$) (Figure 3D). Furthermore, protein expression showed that the dome-shaped strained group displayed higher expression of keratocan, collagen I, and collagen V as compared to both the flat-shaped strained and unstrained groups, as seen on western blots (Figure 3D–F). The protein expression levels of lumican in the dome-shaped strained group were also increased, but not as obvious as other protein levels on western blot (Figure 3C). Taken together, the dome-shaped strain was concluded to have superior effect, regarding the keratocyte markers and the matrix formation markers, as compared to the flat-shaped strain or no strain at all.

Next, we tried to determine the optimal strength of dome-shaped strain to better promote the keratocyte phenotype and ECM formation. The strain in the native cornea is normally in the range of 0%–3%, although it may reach up to 7%\textsuperscript{[32]}. Therefore, we compared the unstrained, 1.5%, 3%, and 6% dome-shaped strain to see if there is any difference among the groups. Real-time PCR analysis revealed that 3% strain significantly enhanced the expression of lumican as compared to the unstrained, 1.5% and 6% strained groups (Figure 4A, $P < 0.001$ to unstrained and 1.5% strain, $P < 0.05$ to 6% strain). Similar results were observed for collagen I (Figure 4C, $P < 0.001$ to unstrained and 1.5% strain, $P < 0.01$ to 6% strain) and collagen

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**Figure 3.** The effects of no strain (unstrained), 3% dome-shaped strain and 3% flat-shaped strain on keratocyte behavior. A) The dome-shaped loading post and the dome-shaped strain to cells in culture. B) The flat-shaped loading post and the flat-shaped strain to cells in culture. The gene and protein expression levels of C) lumican, D) keratocan, E) collagen I and F) collagen V were assessed by real-time PCR and western blot at day 3, respectively. Real-time PCR results are presented as target gene expression/β-actin, normalized to the unstrained group. Results are shown as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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For corneal cells, only some work on tenocytes, ligament fibroblasts, and myocytes has been extensively studied.\(^\text{[34,43–45]}\) For keratocytes, phenotype and ECM formation among the strains tested. The effect of mechanical strain on keratocyte behavior. The gene expression levels of A) lumican, B) keratocan, C) collagen I and D) collagen V were assessed by real-time PCR at day 3. Results are presented as target gene expression/β-actin, normalized to the unstrained group. Results are shown as mean ± SD. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).

Figure 4. The effects of no strain (unstrained), 1.5%, 3%, and 6% dome-shaped strain on keratocyte behavior. The gene expression levels of A) lumican, B) keratocan, C) collagen I and D) collagen V were assessed by real-time PCR at day 3. Results are presented as target gene expression/β-actin, normalized to the unstrained group. Results are shown as mean ± SD. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).

V (Figure 4D, \(P < 0.001\) to all other groups). The 3% strain also significantly upregulated the expression of keratocan compared to the 1.5% strained group but not to the unstrained and 6% strained groups (Figure 4B, \(P < 0.05\) to 1.5% strain, \(P = 0.459\) to unstrained, \(P = 0.588\) to 6% strain). Taken together all results, 3% strain was concluded to be the optimal strength to promote keratocyte phenotype and ECM formation among the strains tested.

It is known that mechanical strain has a direct effect on the cell behavior in culture. The effect of mechanical strain on tenocytes, ligament fibroblasts, and myocytes has been extensively studied.\(^\text{[14,43–45]}\) For corneal cells, only some work has so far been done to investigate the mechanical effects with flat, uniaxial load, which cannot totally reflect the in vivo mechanical environment.\(^\text{[46,47]}\) In the present study, we created a novel static equibiaxial dome-shaped strain which highly replicated the cornea shape and strain. In addition, we tried the strength of strain within the normal range of native cornea (0%–7%). Our results show, for the first time, that 3% dome-shaped strain, which resembles the mechanical environment of native cornea, provides the optimal mechanical stimuli for the keratocytes to maintain their phenotype and ECM deposition in vitro. It not only provides insight into understanding corneal mechanics, but also paves the way for developing new in vitro culture model for corneal cells.

2.4. The Synergistic Effects of Topography and Mechanical Strain on Keratocyte Behavior in a Biomimetic 3D Corneal Model

The topographic effect on cell alignment and the mechanical effect on the gene expression of keratocyte and ECM markers motivated us to study the potential synergistic effects of topographical properties and mechanical strain, i.e., combining the fabricated patterned silk film with the optimal strain. In order to mimic the in vivo corneal environment as closely as possible for these experiments, we created a novel biomimetic 3D corneal model in vitro in a collagen I hydrogel—since collagen I is the main component of the native corneal stroma—which combines the topographical and mechanical cues that we had found to be most similar to the in vivo situation. As depicted in Figure 5A, this model is a collagen-modified, silk fibroin-based construct, together with the treatment of 3% dome-shaped strain. The cells cultured in this 3D model were able to get the topographical cues from the patterned silk fibroin film on the bottom, the mechanical cues of the 3% dome-shaped equibiaxial strain generated by the Flexcell system, and ECM cues within the surrounding collagen I hydrogel environment. Corneal cells cultured on 2D tissue culture plastic (TCP), 2D patterned silk fibroin film (topography, T), and 2D monolayer with 3% dome-shaped strain (mechanical strain, M) were compared to the cells cultured in this 3D biomimetic microenvironment (3D).

Following 3 d of culture, F-actin staining revealed that cells were randomly oriented in the TCP and M group, but aligned along with the groove axis direction in the T and 3D group (Figure 5B). Quantification of cellular orientation angle further showed that it was significantly lower in the T (5.82°) and 3D (15.13°) groups compared with that in the TCP (56.24°) and M (51.26°) groups (Figure 5C, T/3D vs TCP/M: \(P < 0.001\)). We note that the 3D culture environment slightly increased cell direction compared to the patterned silk fibroin film alone (T group, \(P = 0.267\)), indicating that the mechanical strain impacts the cell alignment that was generated by the surface pattern of the silk fibroin film. This is reasonable since the equibiaxial tensile strain produced by the dome-shaped loading post could guide the cells somewhat off the grooves.

The F-actin staining further revealed that cells cultured in the T group were more elongated in comparison to those in the TCP, M, and 3D groups which had a shorter and fusiform morphology as the keratocyte should be (Figure 5B).\(^\text{[14,48]}\) The aspect ratios of cells in the groups with patterned surface (T and 3D) were higher than those in the groups with unpatterned surface (TCP and M) (Figure 5D, T/3D vs TCP/M: \(P < 0.001\); T vs TCP: \(P < 0.001\); 3D vs TCP: \(P = 0.209\)), as expected since the topographical surface is known to potentially enhance cell elongation.\(^\text{[48]}\) Notably, the 3D culture environment decreased this
topographical effect on cell elongation, as evidenced by significantly lower aspect ratio in the 3D group compared to that in the T group ($P < 0.001$).

Additionally, immunofluorescent staining of collagen I and collagen V, the main fibrillary collagen type of the stroma, was performed (Figure 5E). Consistent with the F-actin staining, the
ECM deposition was randomly organized on the flat surfaces of the TCP and M groups, but aligned in the direction of the groove axis on the patterned surfaces of the T and 3D groups. It is difficult to compare the expression levels of collagen from the immunostaining between groups due to the intrinsic fluorescence of silk fibroin films. All these data put together suggest that the 3D corneal model we created better preserves the keratocyte morphology while still maintaining the preferable straight cell alignment and ECM deposition, which is important to corneal transparency, compared to the 2D culture models.

Gene expression levels of the main proteoglycans (lumican and keratocan), and collagen fibrils (collagen I and collagen V) of the corneal stroma, were compared between the four groups. The keratocyte marker lumican was significantly enhanced in the 3D model as compared to the 2D culture conditions (Figure 6A, P < 0.001 to all other groups). Similar results were observed for the gene expression levels of keratocan, another important marker of keratocytes which contributes to corneal transparency (Figure 6B, P < 0.01 to TCP and T, P < 0.05 to M). On the level of ECM markers there was a significant increase in the 3D group as compared to the TCP group (P < 0.01 for collagen I, P < 0.05 for collagen V), and the T group (P < 0.05 for collagen I) (Figure 6C,D). The mechanical strain itself (M group) also increased the genetic expression levels as compared to the groups without strain (TCP and T groups; P = 0.047 for lumican for M vs TCP; P > 0.05 for all other genes and groups). However, the effect was inferior, and in most cases not statistically significant, to the 3D model which combined the mechanical and topographical cues together.

The present study developed a novel 3D biomimetic corneal model which shows promising conditions for in vitro culturing of keratocytes. In this model, the patterned silk fibroin film offered a contact guidance platform for cell alignment, the collagen I hydrogel mimicked a natural ECM microenvironment similar to the native cornea, and the 3% dome-shaped strain reproduced conditions similar to the natural strain of the cornea in the eye. It combined topographical and mechanical cues, and created a highly biomimetic corneal environment favorable to keratocyte growth, thereby significantly enhancing the expression of keratocyte and ECM markers and better maintaining the keratocyte phenotype than the traditional 2D culture conditions do. Previous work by Yang and collaborators reported another interesting 3D multilayered corneal model which combined topographical effects (nanofibers) and chemical effects (serum-free media with insulin). The expression of keratocyte markers were enhanced and, more remarkably, the myofibroblast markers were decreased in their culture model as compared to in the 2D TCP culture, supporting that the 3D culture environment has superior effect on corneal cell lineage than the 2D monolayer culture. Their results also provide useful information for our coming follow-up studies, suggesting that chemical cues, such as culture medium, serum concentration, and biomolecules supplementation, could be further optimized in our 3D model. Furthermore, since our 3D culture model only focuses on the stromal layer, scaffolds with multilayers that support the culture not only of keratocytes, but also of epithelial cells and endothelial cells, can also be applied to better resemble the cell–cell and cell–matrix interactions in native cornea which comprises of epithelium, stroma, and endothelium.

Highly speculative, the 3D biomimetic corneal model here presented might not only be useful in future in vitro cell culture studies, but could potentially also be further exploited and refined in the direction of a tissue-engineered corneal replacement graft for in vivo corneal repair and regeneration. Due to shortage of donor corneal tissue in many places, and the significant immune rejection rate after corneal transplantation, the development of corneal replacement using bioengineering approaches is of great relevance. The 3D silk-based construct we developed has acceptable optical transparency, favorable mechanical strength, and excellent cell biocompatibility, which are the essential properties of an ideal corneal equivalent. It has been reported that patterned silk fibroin film has suitable biocompatibility with the corneal stroma in rabbit. More work needs to be done, including in vivo bio-compatibility tests and in situ animal studies to evaluate the potential of this 3D silk-based construct as a transplant biomaterial for...
cornea repair. Nevertheless, the most apparent application of this model, now and after refinement, is the use for in vitro studies. It is apparent that a suitable in vitro 3D culture model can give a possibility to study human cells in a 3D environment closely mimicking the in vivo conditions, thereby superior in that regard to 2D culture models. Furthermore, it also offers an alternative to the use of ex vivo organ culture models and animal experiments which are expensive and of limited availability.[35]

3. Conclusion

In summary, our study offered a novel biomimetic 3D corneal model by optimizing the surface topography and mechanical strain similar to the microenvironment of native cornea. Compared to traditional 2D culture conditions, this 3D culture model further enhanced the expression of keratocyte and ECM markers, and better maintained the keratocyte phenotype. To our knowledge, it is the first evidence of synergistic effects of surface topography and mechanical strain on keratocyte behavior within a 3D corneal model. It not only provides an in vitro 3D culture model to improve current 2D culture conditions for corneal studies, but also provides valuable insight for the future development of biomimetic tissue-engineered corneal replacement grafts for in vivo applications.

4. Experimental Section

Isolation and Culture of Human Keratocytes: Healthy human corneal tissue was received for research purpose from the corneal biobank at the University Hospital of Umeå, Sweden. The tissue originated from deceased individuals who had chosen, when alive, to donate their corneas postmortem for transplantation and research, according to Swedish law, and the leftover tissue from healthy grafts used for transplant surgery was delivered to the laboratory for research purpose. The Regional Ethical Review Board in Umeå reviewed the study and determined it to be exempted from the requirement for approval (2010-373-31M).

Cell isolation and culture were performed as described previously.[36,37] Corneal samples were scraped using a sterile scalpel to remove any remaining epithelial or endothelial cells, before being washed in sterile Hank’s balanced salt solution (Invitrogen, Carlsbad, CA). The remaining stromal layer was cut into 1–2 mm² pieces with a scalpel and then digested with 2 mg mL⁻¹ collagenase (Sigma, St. Louis, MO) overnight at 37 °C. The suspension was centrifuged and the pellet was cultured in DMEM/F-12 media (Gibco, Carlsbad, CA) supplemented with 2% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and placed in a humidified incubator at 37 °C for 30 min for gelling.

Preparation of Polydimethylsiloxane (PDMS) Molds: The base with the curing agent was mixed at 9:1 ratio using the Sylgard 184 Silicone Elastomer Kit (Dow Corning, Midland, MI). Slowly PDMS was poured over the Petri dish or the diffusion grating (Edmund Optics, Barrington, NJ) with 100 or 600 grooves mm⁻¹ density to prepare the unpatterned or patterned PDMS molds of ≈1.0 mm thickness. After curing at 60 °C for 4 h, PDMS molds were removed from the base and punched into 14 and 33 mm diameter disks.

Preparation of Silk Fibroin Films: 180 or 940 μL of 4% silk fibroin solution was added onto each 14 or 33 mm unpatterned/patterned PDMS mold and allowed to dry overnight. Once dried, the silk fibroin films were placed in a water-filled vacuum desiccator (Sanpatec Corp., Osaka, Japan), and a water-annealing procedure was performed. In this study, different water-annealing time (2–24 h) was performed and the optimal time was chosen based on its effects on the film stability, cell proliferation, etc. The 14 mm film was used for the 24-well plate, and the 33 mm film was used for the 6-well plate. The silk fibroin films were stored at room temperature before use.

Mechanical Stimulation: Cells were seeded on Bioflex 6-well plate (Flexcell International Corporation, Burlington, NC), and were left to adhere overnight and then placed on a loading post of 25 mm diameter, either with a corneal (dome) shape or with a flat shape loading post. In the Flexcell Tension System, the membranes were then pulled downward by vacuum suction which causes the membranes to stretch across the loading post, and the adherent cells experience equibiaxial strain. The cells were kept under 1.5%, 3%, or 6% strain consistently, statically, throughout the experiments with 3.5 mL of culture media (n = 3 per group). The control group (unstrained) was cultured in the same conditions but without being exposed to strain.

3D Corneal Model: Cells were seeded on the 33 mm 600 grooves mm⁻¹ silk fibroin film which was placed and fixed with tape in the Bioflex 6-well plate (Flexcell International Corporation, Burlington, NC), and were left to adhere overnight to mimic the in vivo corneal microenvironment, rat tail collagen type I hydrogels (Thermo Fisher Scientific, Waltham, MA) was prepared according to the manufacturer’s instructions. In each well, 2 mL of collagen hydrogels (2.4 mg mL⁻¹) was added upon the cell-seeded silk fibroin film and incubated at 37 °C for 30 min for gelling. After a firm gel was formed, the plate was placed on a 25 mm diameter, dome-shaped loading post. The cells were kept under 3% strain with 3.5 mL of culture media.

MTS Cell Proliferation Assay: 1.2 × 10⁴ cells were seeded on each 14 mm silk fibroin film and cultured for the defined time periods (n = 4 per group). Cell proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI) according to the previous study.[38] The absorbance of the culture medium was measured at 490 nm using a Synergy HT plate reader (BioTek, Winooski, VT) in the wavelength range 350–850 nm with 5 nm intervals. The transparency of the films was calculated using the formula (n = 4 per group)

\[
\text{Absorbance} = -\log(\%\text{transmittance}/100) \tag{1}
\]

SEM Preparation and Imaging: Samples were fixed using 2.5% glutaraldehyde in 0.1 M Sodium Cacodylate buffer at 4 °C overnight and rinsed with PBS for three times. Samples were dehydrated in increasing concentrations of ethanol and were critically point-dried. They were mounted on aluminum stubs and coated with 2.5 nm Iridium, then viewed under a Carl Zeiss Merlin Field Emission Scanning Electron Microscope with SmartSEM V5.05 software.

Cell Alignment Analysis: Three samples per group were fixed in 3.7% (v/v) paraformaldehyde, and permeabilized with 0.1% Triton X-100. F-actin staining was performed using BODIPY FL Phallacidin (Life Technologies, Carlsbad, CA) for 30 min. Samples were mounted in ProLong Diamond Antifade Mountant with DAPI
per sample were examined and a minimum of 50 cells were measured. Five individual 20× images per sample were examined and a minimum of 50 cells were measured per sample.

**Cellular Aspect Ratio Analysis:** Three samples per group were stained with F-actin as described above. The aspect ratio of the cells was measured using the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD) as previously described. Complementary lines of interest were drawn across the length and width within the cell borders to measure the extremities of the cell. The ratio of length: width was used to determine the cellular aspect ratio. Five individual 20× images per sample were examined and a minimum of 50 cells were measured per sample.

**Immunofluorescence Staining:** Immunofluorescence staining was performed as described previously. Three samples per group were fixed with 3.7% (v/v) paraformaldehyde, permeabilized with 0.1% Triton X-100, then blocked with 1:20 diluted normal serum. Samples were incubated with the primary antibody overnight at 4 °C. After washing, secondary antibody labelled with tetramethylrhodamine (TRITC) was added together with BODIPY FL Phallacidin (Life Technologies, Carlsbad, CA) and DAPI (Thermo Fisher Scientific, Waltham, MA) diluted in PBS for 30 min. Finally, samples were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis. All antibodies used are summarized in Table 1.

**RNA Isolation and Real-Time PCR:** Total cellular RNA isolation and real-time PCR analysis was performed as described previously. Total RNA was isolated from keratocytes from each group (n = 3 per group) by RNeasy/miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and reverse transcribed into cDNA by using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). To determine the gene expression, TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) were used. cDNA transcribed from 40 ng of RNA was run in duplicates by ViIA 7 Real-Time PCR system, and analyzed with ViIA 7 Software (Applied Biosystems, Carlsbad, CA). Results are presented as target gene expression/β-actin normalized to the control group. All probes used for real-time PCR (Applied Biosystems, Carlsbad, CA) are summarized in Table 2.

**Western Blot:** Cells in triplicate were collected and combined into one sample for each condition. Samples were lysed in Radioimmunoprecipitation Assay (RIPA) lysis buffer, supplemented with protease inhibitor (Sigma, St. Louis, MO) and diluted in Laemmli buffer (Bio-Rad, Hercules, CA) supplemented with β-mercaptoethanol. After boiling the samples, equal total proteins were loaded into each well of a pre-made gels of 12% (Mini-PROTEAN TGX, Bio-Rad, Hercules, CA) and ran at 160 V for ~45 min. Subsequently, proteins were transferred to a polyvinylidene fluoride transfer membrane (Santa Cruz, Dallas, TX) membrane for 60 min at 100 V. Membranes were blocked for 1 h in room temperature before primary antibody was added and incubated at 4 °C overnight. After washing, the membranes were exposed to the secondary antibody (conjugated with horseradish peroxidase, HRP) for 1 h and then to the enhanced chemiluminescence solution (GE healthcare, Little Chalfont, UK) for 5 min in room temperature. The membranes were developed using Odyssey Fc imaging system (LI-COR, Lincoln, NE). All antibodies used are summarized in Table 1.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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