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Keratocyte Mechanobiology

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Abstract

In vivo, corneal keratocytes reside within a complex 3D extracellular matrix (ECM) consisting of highly aligned collagen lamellae, growth factors, and other extracellular matrix components, and are subjected to various mechanical stimuli during developmental morphogenesis, fluctuations in intraocular pressure, and wound healing. The process by which keratocytes convert changes in mechanical stimuli (e.g. local topography, applied force, ECM stiffness) into biochemical signaling is known as mechanotransduction. Activation of the various mechanotransductive pathways can produce changes in cell migration, proliferation, and differentiation. Here we review how corneal keratocytes respond to and integrate different biochemical and biophysical factors. We first highlight how growth factors and other cytokines regulate the activity of Rho GTPases, cytoskeletal remodeling, and ultimately the mechanical phenotype of keratocytes. We then discuss how changes in the mechanical properties of the ECM have been shown to regulate keratocyte behavior in sophisticated 2D and 3D experimental models of the corneal microenvironment. Finally, we discuss how ECM topography and protein composition can modulate cell phenotypes, and review the different methods of fabricating in vitro mimics of corneal ECM topography, novel approaches for examining topographical effects in vivo, and the impact of different ECM glycoproteins and proteoglycans on keratocyte behavior.

Keywords

Corneal keratocytes; Corneal Stroma; Cell Mechanics; Mechanobiology; Extracellular Matrix

1. Introduction

Many cells have mechanical phenotypes that are essential for normal tissue function. This is particularly evident during processes like embryonic development and wound healing, where

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cells must generate mechanical forces in order to migrate, contract, and rearrange their local ECM to repair injuries or form new tissues (Mammoto and Ingber, 2010; Miller and Davidson, 2013). While biochemical cues, such as diffusible growth factors, are indispensable to these processes, cells also experience highly dynamic changes in stiffness and mechanical force, which also regulate their behavior (Discher et al., 2005; Jaalouk and Lammerding, 2009). Cells are connected to one another and to the extracellular matrix (ECM) via a variety of different transmembrane protein complexes that couple the actin cytoskeleton to structures outside of the cell (Alberts et al., 2015). These include integrins, which bind to specific ECM molecules and constitute the basis for focal adhesion (FA) complexes, as well as cadherins, which form homotypic bonds to couple mechanically the actin cytoskeletons of neighboring cells. Cells exert mechanical forces via these different transmembrane proteins and transduce mechanical inputs into changes in biochemical signaling (Leckband et al., 2011; Ross et al., 2013), which can lead to dramatic changes in cell phenotype.

Keratocytes reside between the collagen lamellae in the cornea, and secrete the ECM components necessary to maintain normal corneal structure and transparency (Chakravarti et al., 2000; Funderburgh et al., 2003; Hassell and Birk, 2010). In adult corneal tissue, resting keratocytes are mechanically quiescent; they do not express stress fibers or generate substantial contractile forces (Garana et al., 1992; Jester et al., 1994; Lakshman et al., 2010; Stramer et al., 2003). Following injury, surgery or other insults, corneal keratocytes can become activated by growth factors and other cytokines present in the wound environment, and transform into a fibroblastic repair phenotype (Garana et al., 1992; Stramer et al., 2003). Corneal fibroblasts proliferate, develop intracellular stress fibers, migrate into wounds and can reorganize the ECM through the application of mechanical forces. In certain wound types, the presence of transforming growth factor beta-1 (TGF β 1) can induce transformation of corneal fibroblasts to myofibroblasts, which express alpha smooth muscle actin (α -SMA), generate stronger tractional forces on the matrix, and synthesize a disorganized fibrotic ECM (Blalock et al., 2003; Jester et al., 1999). Together these processes can impact visual acuity by altering corneal shape and reducing transparency due to increased light scattering by both cells and the newly synthesized ECM (Boote et al., 2012; Dupps and Wilson, 2006; Jester et al., 2012; Moller-Pedersen et al., 1998; Moller-Pedersen et al., 2000; Ruberti et al., 2011).

In addition to growth factors and other biochemical factors, ECM stiffness, topography and/or protein composition have all been shown to modulate keratocyte differentiation, contractility and patterning. In the cornea, shifts in the distribution of tension can be induced by stromal injury or surgery (Dupps and Wilson, 2006; Ruberti et al., 2011). Keratoconus also leads to changes in stromal structural and mechanical properties, including reduced mechanical stiffness (Ali et al., 2014; Andreassen et al., 1980; Edmund, 1989; Morishige et al., 2007) and thinning of the central cornea which results in redistribution of stromal tension (Ambekar et al., 2011). In contrast, treatment of keratoconus with UV cross-linking increases corneal stromal rigidity (Beshtawi et al., 2013). Thus, changes in stromal structure and mechanical properties can potentially modulate corneal keratocyte responses to a range of clinical conditions (Winkler et al., 2011). In this article, we review how both biochemical and biophysical factors can regulate keratocyte differentiation and mechanical activity at the cellular and sub-cellular level. We include studies on all three of the primary keratocyte

phenotypes observed in vivo: quiescent corneal keratocytes, activated corneal keratocytes (corneal fibroblasts), and transformed corneal keratocytes (corneal myofibroblasts).

2. Biochemical regulators of corneal keratocyte mechanical activity

Peptide growth factors present in the cornea and tear film, such as insulin growth factor (IGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin 1 alpha (IL-1 α), hepatocyte growth factor (HGF) and TGF β (Arnold et al., 1993; Kim et al., 1999; Miyagi et al., 2018a; Miyagi et al., 2018b; Musselmann et al., 2008; Tuominen et al., 2001) are postulated to play an important role in modulating the keratocyte phenotype during corneal wound healing. In cell culture, these growth factors differentially regulate keratocyte proliferation, cytoskeletal organization and ECM synthesis (Beales et al., 1999; Chen et al., 2009; Etheredge et al., 2009; Funderburgh et al., 2001; Jester et al., 1994; Jester and Chang, 2003; Jester et al., 2002; Long et al., 2000; Maltseva et al., 2001). These changes in cell phenotype are often associated with changes in cell mechanical behaviors. For example, under serum-free conditions, IGF and PDGF BB stimulates elongation of corneal keratocytes within 3-D matrices, without stimulating significant force generation. In contrast, treatment with TGF β increases keratocyte contractility, as indicated by stress fiber formation and significant matrix compaction; and this transformation is increased in stiffer ECM environments (Lakshman and Petroll, 2012). Changes in cell mechanical behavior in response to soluble growth factors and/or changes in the biophysical environment are regulated by a number of complex signaling pathways. In some cases, forces acting at the cell membrane induce changes in nuclear structure directly, since the actin cytoskeleton is coupled to the nucleus via Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes (Isermann and Lammerding, 2013), but here we focus on changes in the activity of key signaling cascades downstream of integrins – specifically the Rho and Hippo signaling pathways – which have been shown to modulate cell mechanical differentiation in response to growth factors, as well as changes in both ECM stiffness and topography.

2.1. Rho/Rac regulation of the sub-cellular pattern of force generation

The Rho-family of small GTPases such as Rho, Rac, and Cdc42 are key regulators of mechanical activities in many cell types, and often mediate the response to growth factors and other cytokines (Hall, 2005; Hodge and Ridley, 2016; Warner et al., 2019). Rho binds to and activates Rho kinase, which inhibits myosin light chain (MLC) phosphatase, resulting in elevated MLC phosphorylation and increased cell contractility (Amano et al., 1996; Amano et al., 2010; Parizi et al., 2000). Activation of Rho generally stimulates the formation of stress fibers and focal adhesions on rigid substrates (Jester and Chang, 2003; Rottner et al., 1999).

In 3-D culture models, insights into dynamic cell-ECM interactions can be made by combining time-lapse differential interference contrast (DIC) imaging (to visualize collagen fibrils and map their displacement) with live-cell fluorescent imaging (to track reorganization of GFP-tagged cytoskeletal proteins) (Petroll and Ma, 2003; Petroll et al., 2008; Vishwanath et al., 2003). Local collagen matrix organization can also be directly visualized using confocal reflection imaging, which generates high resolution images of

collagen fibers from within 3-D matrices without the need for exogenous labeling (Friedl, 2004). Both of these approaches have been used to study the impact of Rho GTPases on the mechanical behavior of corneal keratocytes and fibroblasts. Inhibiting Rho kinase induces rapid elongation of corneal fibroblasts, formation of dendritic cell processes, a reduction in cell-induced tension on the matrix (Figure 1), as well as a decrease in local matrix compaction and alignment (Kim et al., 2006; Vishwanath et al., 2003). Rho kinase has also been shown to mediate specific keratocyte growth factor responses, such as fibroblastic transformation in response to basic fibroblast growth factor (FGF2) and myofibroblast transformation in response to TGF β (Chen et al., 2009; Lakshman and Petroll, 2012; Yamamoto et al., 2012).

The role of Rho kinase in regulating corneal fibroblast migration mechanics in 3-D matrices has also been investigated, using a nested matrix model that facilitates imaging of cell-matrix interactions during cell translocation. During migration, serum-cultured corneal fibroblasts generated significant tractional forces at the leading edge, as indicated by displacement and compaction of collagen (Zhou and Petroll, 2010). Following Rho kinase inhibition, cells became more elongated and extended dendritic processes at random orientations, and the rate of cell migration was significantly reduced. Furthermore, the normal mechanical sequence typical of mesenchymal cell migration (extension, contraction, tail retraction) was not observed, consistent with other studies indicating that Rho kinase mediates mechanical coordination between the front and rear of the cell (Amano et al., 2010; Ridley, 2015).

In contrast to Rho, Rac generally stimulates cell spreading and migration, via the creation of smaller focal complexes and actin polymerization (Demali and Burridge, 2003; Rottner et al., 1999; Sander et al., 1999; Svitkina and Borisy, 1999; Totsukawa et al., 2000). Rac can be activated by platelet derived growth factor BB (PDGF BB), which also stimulates cell spreading and migration (Andresen et al., 1997; Grinnell et al., 2006). In time-lapse studies of corneal fibroblasts in 3-D matrices, PDGF induced a shift in the distribution of forces from the center to the periphery of corneal fibroblasts during cell spreading (Petroll and Ma, 2008; Petroll et al., 2008). Relaxation (decompression) of the ECM was observed along the cell body, whereas tractional forces were generated on the collagen by extending cell processes, with compression at the base of the pseudopodia (Figure 2). Inhibition of Rho kinase or myosin II promoted significant ECM relaxation, but small displacements of collagen fibrils continued to be detected at the pseudopodial tips that may be both ROCK and myosin II-independent.

For corneal keratocytes maintained in serum-free conditions (in which the level of Rho activation is low), only limited global and local ECM reorganization is induced by PDGF BB (Lakshman and Petroll, 2012). Under these conditions, only small, transient matrix deformations are produced during migration, and matrix reorganization is significantly reduced as compared to serum or TGF β 1, in which Rho predominates (Figure 3) (Kim et al., 2012; Zhou and Petroll, 2014). Following corneal injuries that penetrate the stroma, cellular force generation is needed to facilitate wound closure and restore the mechanical integrity of the cornea. However, following refractive surgery or corneal cross-linking procedures which create an area of cell death within the stromal ECM, it is better to minimize cellular force

generation and fibrosis during stromal repopulation, since these processes can effect ECM structure and transparency (Dupps and Wilson, 2006; Moller-Pedersen et al., 2000; Netto et al., 2005). The balance between Rho and Rac activation is likely important in defining these distinct cellular phenotypes.

2.2. YAP/TAZ regulation of keratocyte mechanical behavior

The activity of YAP and TAZ, two downstream effectors of the Hippo signaling pathway, have also been linked to changes in mechanical force and ECM stiffness (Dupont et al., 2011). When Hippo signaling is activated, both of these transcription factors are phosphorylated by LATS1/2, which sequesters them to the cytoplasm where they are tagged for degradation. However, when Hippo signaling is inactive, YAP and TAZ are dephosphorylated and translocate to the nucleus, where they bind with TEAD and promote the expression of downstream target genes (Totaro et al., 2018). Numerous studies have reported YAP and TAZ localized to the nucleus on stiff substrata, but sequestered in the cytoplasm in compliant microenvironments (Halder et al., 2012). YAP/TAZ activity is thought to be related to ECM rigidity via changes in signaling downstream of focal adhesions, likely involving the Rho pathway (Totaro et al., 2018).

Despite its clear role in the myofibroblastic activation of other cell types (Caliari et al., 2016; Liu et al., 2010; Ma et al., 2017), few studies have assayed directly the effect of YAP/TAZ signaling on the myofibroblastic differentiation of corneal keratocytes. Using human corneal fibroblasts cultured in serum-containing media, Western blots have suggested that treatment with TGF β 1 can alter the phosphorylation state of both YAP and TAZ, and siRNA knockdown of TAZ expression has been shown to increase the relative expression of α -SMA mRNA (Muppala et al., 2019). Somewhat surprisingly, siRNA knockdown of YAP had no discernible effect on α -SMA expression, suggesting the possibility of distinct roles for these two different Hippo effector proteins during the myofibroblastic differentiation of corneal fibroblasts. It remains unclear, however, how the nuclear localization of YAP and TAZ, as well as the expression of their downstream targets, are modulated by treatment with TGF β 1, and whether or not both of these transcription factors are sequestered to the cytoplasm in quiescent keratocytes that have not been exposed to serum. Additional work in this area is warranted.

3. Feedback from ECM mechanical properties regulates corneal keratocyte behavior

During development, tissues often undergo dramatic changes in mechanical properties, as embryonic tissues are often several orders of magnitude softer than their counterparts in the adult organism (Wozniak and Chen, 2009). These changes in tissue stiffness can have profound effects on the differentiation of progenitor cell populations (Bellas and Chen, 2014). Stem cells, for instance, undergo lineage-specific differentiation when cultured on substrata that approximate the stiffness of native tissue (Murphy et al., 2014). These assays often involve the use of polyacrylamide (PA) substrata, a synthetic material with tunable stiffness, which can be functionalized with different ECM proteins to enable cell attachment (Pelham and Wang, 1997; Wang and Pelham, 1998). Using such a system, pluripotent

mesenchymal stem cells have been shown to differentiate into either neurogenic, myogenic, or osteogenic lineages, depending on the stiffness of the underlying substratum (Engler et al., 2006). Spatial control of the PA polymerization rate, through the use of photomasks and specific photo-initiators, can also be used to generate stiffness gradients of defined magnitude within individual PA gels (Tse and Engler, 2010), enabling the formation of defined spatial gradients in cell differentiation (Tse and Engler, 2011).

Tissue repair and wound healing also involve significant changes in ECM stiffness (Hinz, 2007), and numerous studies have used tunable PA (or silicone) substrata to modulate the myofibroblastic differentiation of fibroblasts derived from several tissues, including skin, lung, and cornea (Dreier et al., 2013; Goffin et al., 2006; Marinkovic et al., 2012). These compliant substrata can also be combined with microcontact printing (Tien et al., 2002) to create adhesive ECM islands of defined size and stiffness (Goffin et al., 2006). Differently-sized, micropatterned islands of fibronectin, for instance, have been used to constrain the size of focal adhesion (FA) complexes within cultured fibroblasts on flexible silicone substrata. Constraining FA size below $\sim 6 \mu\text{m}$ was sufficient to disrupt the observed stiffness-dependent differences in myofibroblastic differentiation (Goffin et al., 2006).

3.1. Studies on keratocytes using flexible 2-D Substrates

Mechanical interactions between cells and ECM influence profoundly the behavior of corneal keratocytes in the stromal compartment (Petroli and Miron-Mendoza, 2015), especially following an injury, since changes in tissue stiffness often accompany wound repair in the cornea (Raghunathan et al., 2017; Thomasy et al., 2014). Extensive ECM remodeling underlies these changes in mechanical properties, but it is not always clear how ECM stiffness, as distinct from ECM composition and topography, modulates directly the behavior of corneal keratocytes. In 2D culture, the use of synthetic PA hydrogels allows substratum stiffness to be de-coupled from ECM composition and topography (Fischer et al., 2012; Pelham and Wang, 1997; Wang and Pelham, 1998) (Lee et al., 2012).

Very few studies have investigated the effects of ECM stiffness on keratocyte behavior in 2D culture, but functionalized PA gels have been used previously to show that changes in substratum stiffness can modulate the expression of α -SMA in corneal fibroblasts cultured in serum-containing media (Dreier et al., 2013; Thomasy et al., 2018). Somewhat surprisingly, stiff substrata were found to promote α -SMA expression in both the presence and absence of exogenous TGF- β 1, though at decidedly higher levels in the TGF- β 1-treated cells. The stiffness-dependent increase in α -SMA expression was diminished by treatment with either hepatocyte growth factor (HGF) (Miyagi et al., 2018a) or latrunculin B (Thomasy et al., 2018). In each of these studies, however, it is not clear how the mechanical phenotype (e.g., contractility, traction forces) of the cultured cells might be modulated by changes in substratum stiffness, nor how corneal fibroblasts sense the stiffness of the underlying substratum. To further understand how changes in ECM stiffness regulate keratocyte activation and contractility, we cultured primary rabbit corneal keratocytes on PA substrata of varying stiffness and maintained the cells in defined serum-free culture conditions to promote a quiescent keratocyte phenotype in the absence of exogenous growth factors (Maruri et al., In Press). Using this system, we showed that changes in ECM stiffness

can modulate the morphology, cytoskeletal organization, and subcellular pattern of force generation in keratocytes treated with TGF- β 1 (Figure 4). Interestingly, however, keratocytes cultured in the absence of TGF- β 1 showed no stiffness-dependent phenotypes, suggesting that a stiff microenvironment alone is insufficient to promote keratocyte transformation (Maruri et al., In Press). The mechanotransductive pathways that underlie these changes in keratocyte behavior, however, remain unclear. Prime targets include both the Rho and Hippo signaling pathways. Going forward, the use of PA substrata in 2D culture offer a promising experimental platform to determine how keratocytes sense changes in ECM stiffness and transduce these mechanical inputs into changes in cell behavior.

3.2. Studies on keratocytes using 3-D culture models

Studies using corneal fibroblasts in 3-D culture have also shown an interdependence between cell and matrix mechanics. Like most cell types, corneal fibroblasts tend to align and compact collagen parallel to the axis of greatest effective stiffness under anisotropic conditions (Karamichos et al., 2007). Using a novel bioreactor in which collagen substrates could be mechanically loaded along a single axis, Ruberti and coworkers demonstrated that mechanical anisotropy induced long-range cell and ECM alignment, with increased stability as compared to isotropic substrates (Zareian et al., 2016).

Cell migration is also be influenced by substrate stiffness. In 3-D matrices with a gradient in collagen density, fibroblasts have been shown to migrate towards the denser, and thereby stiffer, region of the matrix, a phenomenon called “durotaxis” (Hadjipanayi et al., 2009; Lo et al., 2000). To evaluate the effect of matrix mechanical properties on corneal keratocyte motility, migration has been compared in constrained (higher effective stiffness) and unconstrained (lower effective stiffness) nested matrix constructs (Kim et al., 2012). Consistent with previous results using dermal fibroblasts (Grinnell et al., 2006), invasion of keratocytes cultured in serum (i.e. corneal fibroblasts) was significantly reduced in unconstrained matrices. Interestingly, the dependency of corneal fibroblast migration on matrix stiffness was eliminated when Rho kinase was inhibited. Furthermore, migration of keratocytes cultured in serum-free media supplemented with PDGF BB, which have low levels of contractility, was also unaffected by changes ECM constraints (Kim et al., 2012). This is consistent with the concept that durotaxis only regulates the migration of contractile cells (Grinnell and Petroll, 2010).

3.3. Tensional homeostasis

Time-dependent changes in ECM stress can also have a profound impact on cell mechanical behavior. Using a tensioning 3-D culture system in which forces could be measured while strain is applied, Brown and coworkers demonstrated that stretching of collagen matrices seeded with dermal fibroblasts resulted in a rapid increase in the matrix tension (Brown et al., 1998), which was immediately followed by a gradual cell-dependent reduction in force back toward the baseline level. In contrast, decreasing strain on the matrix resulted in an initial loss of tension, followed by a cell-dependent increase back to the baseline level. In both cases, cells responded in a way that maintained “tensional homeostasis” (constant tension) in their environment (Freyman et al., 2002).

Dynamic changes in ECM tension have also been shown to impact corneal fibroblast behavior. Experiments using cyclic stretching of 2-D substrates suggests that stretching magnitude determines whether the cornea undergoes matrix degradation or synthesis, by changing the balance between MMP and TIMP secretion by corneal fibroblasts (Liu et al., 2014). The impact of dynamic changes in local ECM tension has also been investigated, by using micropipettes to displace collagen fibrils near a cell (Petroli and Ma, 2008; Petroli et al., 2004). In these studies, reducing effective local 3-D collagen stiffness in front of a cell resulted in rapid cellular shortening, with corresponding ECM compression along the cell body (Figure 5A–D). This initial contraction was likely due to the release of pre-existing cellular contractile forces, and was blocked by Rho kinase inhibition. Following this initial contraction, pseudopodial spreading was observed at both ends of the cell, and collagen was pulled inward during this secondary spreading. FEM analysis demonstrated that following the initial loss of ECM tension in front of cells induced by the needle push, the secondary spreading response of fibroblasts partially re-established baseline tension, a pattern which is consistent with the tensional homeostasis model. Interestingly, compressing the ECM perpendicular to the long axis of corneal fibroblasts did not elicit any changes in cell morphology or mechanical activity in this study, presumably because cellular forces are not aligned in this direction (Figure 5E–H). Overall, durotaxis and tensional homeostasis appear to regulate fibroblast behavior in response to sustained and transient changes in ECM tension, respectively (Brown et al., 1998; Hadjipanayi et al., 2009; Mizutani et al., 2004; Petroli et al., 2004).

4. The impact of ECM topography and composition on corneal keratocyte behavior

Topographical cues in the local microenvironment are key modulators of cell behavior during both physiological and pathological processes due to the fact that (i) a majority of cell types in the body are anchorage-dependent and (ii) most tissue architectures (e.g., bone, cornea, tendon, myocardium) have regular and anisotropic fibrous structures. Topographical length scales in the body can range from the nanometer (nm) scale diameter of an individual collagen fibril (~30 nm), to the micron scale dimensions (~100–200 μ m) of intestinal crypts. Topographical cues can provide both spatial and mechanical signals, and have been shown to modulate a broad range of cell behaviors such as adhesion (Teixeira et al., 2003; Koh et al., 2010), morphology (Kiang et al., 2013; Whited and Rylander, 2014), proliferation (Chaudhuri et al., 2016; Muhammad et al., 2015), differentiation (Dang and Leong, 2007; Metavarayuth et al., 2016), migration (Diehl et al., 2005), polarity (Ferrari et al., 2010; Ventre et al., 2014), and intracellular signaling (Lou et al., 2018; Dalby et al., 2003). The impact of topographical cues are dependent upon a number of variables such as their dimensions, structure (i.e. pillars, fibers, pits, cavities), stiffness, and the type of cell interacting with the topographical cue. For example, it has been shown that the alignment and migration of endothelial cells (ECs), fibroblasts, and smooth muscles cells (SMCs) on grooved substrates depends upon the dimensions of the grooves (i.e. height and depth), and that fibroblasts had a stronger response, in terms of alignment and migration, than ECs or SMCs. (Biela et al., 2009) Likewise diabetic endothelial cells had reduced functional responses (e.g. uptake and metabolism of oxidized low density lipoprotein, decreased

immune activation) to substrate topography when compared to healthy endothelial cells. (Cutiongco et al., 2018)

4.1. Regulation of keratocyte patterning by ECM topography

4.1.1. In vitro models—The corneal stroma is a highly structured environment that is enriched in both topographical and compositional cues. The topographical signals that keratocytes experience are primarily related to the unique lamellar arrangement of collagen fibrils, while the compositional cues in vivo are due to the proteins (i.e. collagen), growth factors, and proteoglycans present in the native cornea. To mimic the two-dimensional (2D) topography of the corneal stroma, a number of different approaches using both synthetic and natural materials and different culture conditions (i.e. time, media, passage number) have been employed which have provided new insights into keratocyte biology. Here, we focus primarily on those studies that utilized the more popular methods of creating surfaces of defined topography for cell culture, including photolithography, electrospinning, and microfluidic deposition (Table 1).

Initial studies on the role of topography on keratocyte behavior employed photolithography generated substrates in the form of ridges and grooves. For example, culturing human keratocytes on synthetic silicone substrates containing grooves and ridges (400 – 4000 nm) in serum containing media caused them to strongly align in the direction of the anisotropic patterns. In addition, the formation of focal adhesions and stress fibers by keratocytes was reduced on 70 nm-wide ridges when compared to micron-size patterns or smooth substrates (Teixeira et al., 2004). Similar patterns of alignment were observed when human keratocytes were cultured on collagen micropatterns fabricated by deposition of a ~40 µm thick film of collagen on polydimethylsiloxane (PDMS) substrates containing micron sized grooves (Vrana et al., 2008). Moreover after 7 days of culture, the secretion of extracellular matrix molecules (i.e. type I collagen and keratan sulfate) by keratocytes was also oriented parallel to the micropatterns. In comparison, when rabbit keratocytes were cultured on collagen coated grooves and ridges in the absence of serum, orientation and elongation of the keratocytes was only observed on pitches 800 nm or greater (Figure 6), which suggested that the lower limit of the tactile spatial acuity of keratocytes was higher than an individual collagen fibril (~d = 30 nm) (Pot et al., 2010). In contrast to these results, when human corneal stromal stem cells (hCSCs) were cultured on polycarbonate transwell filters containing parallel grooves with a spacing of 200–300 nm and 0.45 µm diameter pores, the hCSCs not only aligned parallel to the grooves, but also secreted extracellular matrix of aligned collagen fibrils that contained type I and V collagen and exhibited a cornea-like lamellar structure (Karamichos et al., 2014). This suggests that sub-micron topographical structures could also promote keratocyte alignment. To investigate the interplay between substrate stiffness, surface chemistry, and topography on keratocyte morphology, human keratocytes were cultured on ridges and grooves made from PDMS (hard and hydrophobic) or chitosan (soft and hydrophilic) (Koo et al., 2011). Keratocyte alignment was observed on both PDMS and chitosan substrates with gratings smaller than 1 µm wide, and the proliferation of keratocytes decreased as the grating size decreased on both substrates. In contrast the keratocytes elongated to a greater extent on PDMS as compared to chitosan, suggesting an interplay between stiffness and topography on keratocyte behavior.

A second popular technique that has been utilized to study the effects of topography on keratocyte behavior is electrospinning, due to its ability to form aligned fibers with well-controlled diameters in the nm to sub-micron range. For example, Yan and colleagues fabricated fibrous scaffolds with sub-micron sized diameters with either randomly oriented ($d = 955 \pm 56$ nm) or aligned ($d = 856 \pm 42$ nm) fibers by electrospinning a blend of gelatin and poly-L-lactic acid (PLLA) (Yan et al., 2012). When rabbit keratocytes were cultured in serum free medium on aligned fibers the cells had a bipolar morphology and were significantly elongated in the direction of the gelatin-PLLA fibers, while keratocytes on randomly oriented fibers displayed an unordered growth pattern. Similarly, Stafiej and colleagues utilized electrospinning to fabricate matrices of random and aligned nanofibers made from hydrophobic polycaprolactone (PCL) as well as blends of PCL with the hydrophilic elastomer poly(glycerol sebacate) (PGS), or the natural polycationic linear polysaccharide chitosan polymer (Stafiej et al., 2017). Despite the wide range of fiber diameters (108 – 536 nm) produced, when a human corneal fibroblast cell line was cultured on these matrices (i.e. PCL, PCL/PGS blend, PCL/Chitosan blend) there was oriented and elongated cell growth on all the aligned fiber substrates, but unstructured growth on the random fiber matrices. No differences in cell adhesion or metabolic activity were observed on the different polymer blends, suggesting that the surface chemistry of the fibers had minimal impact. To investigate the role of topography in inducing the secretion of native corneal stroma extracellular matrix (ECM), hCSCs were cultured on both random and aligned poly(ester urethane) urea fibers in the presence of keratocyte differentiation medium (Figure 7) (Wu et al., 2012). Although hCSCs cultured on both the random and aligned fibers deposited a dense collagenous matrix that was abundant in type I collagen, and contained collagen V, collagen VI, keratan sulfate, and keratocan, only the cells on the aligned fibers were induced to elongate and orient in the direction of the PEUU fibers. In addition, only the deposited ECM on the aligned PEUU showed collagen fibrils with uniform diameter and regular interfibrillar spacing, similar to the native cornea stroma.

Given the importance of aligned type I collagen fibrils in defining the topography of collagen lamella in the corneal stroma, as well as in tendons and vascular tissue, a number of techniques have been developed to form these fibrillar structures. Some of the techniques developed include molecular crowding (Saeidi et al., 2012), spin coating (Saeidi et al., 2011), magnetic fields (Torbet et al., 2007), and microfluidic deposition (Koster et al., 2007; Lanfer et al., 2008; Lee et al., 2006; Saeidi et al., 2009). Although a wide variety of cell types (e.g. endothelial cells (Huang et al., 2013), smooth muscle cells (Nguyen et al., 2016), cancer cells (Wang et al., 2015), and mesenchymal stem cells (Lanfer et al., 2009)) have been cultured on these substrates, the behavior of keratocytes on type I aligned collagen fibrils has been understudied. Recently our group has reported the development of a high-throughput microfluidic method to deposit aligned fibrils of type I collagen onto glass coverslips (Kivanany et al., 2018b; Lam et al., 2019). Integration of these substrates into a PDMS microwell culture system allowed us to perform high-resolution imaging of keratocyte interactions with aligned type I collagen fibrils. When primary rabbit keratocytes were cultured, in the presence of platelet-derived growth factor (PDGF), on substrates coated with varying densities of aligned collagen fibrils, we observed that keratocyte alignment was fibril density dependent. On substrates containing a low fibril density keratocyte orientation

was random, however on substrates containing a high fibril density the keratocytes preferentially aligned parallel to the aligned collagen fibrils (Lam et al., 2019). In a second study, we used time-lapse imaging to assess the dynamic interactions between primary rabbit keratocytes and aligned collagen fibrils during wound repopulation following an in vitro freeze injury model (Figure 8) (Kivanany et al., 2018b). We observed that when keratocytes were cultured in serum free media they would generally spread parallel to the aligned collagen fibrils, however the degree of keratocyte co-alignment with the type I collagen fibrils increased when keratocytes were cultured in the presence of PDGF or TGF β 1. These results, along with those discussed previously, suggest that keratocytes don't respond to topography independent of other variables, but rather their behavior is dependent upon a number of experimental variables (i.e. topography size/shape, cell-type, composition of the culture medium).

4.1.2. In vivo studies—Using volume scanning electron microscopy, Quantock, Jester and coworkers demonstrated that dendritic keratocyte processes are closely associated with orthogonally arranged stromal collagen fibrils in the developing chick cornea (Young et al., 2014), and changes in keratocyte patterning appearing at E9 preceded and predicted later changes in collagen organization. These studies suggest a role for keratocytes in patterning of the embryonic stromal tissue; however, a more recent study suggests that collagen can organize in the absence of cells at earlier time points (E3) (Young et al., 2019).

Following transcorneal freeze injury in the adult rabbit, the alignment of corneal fibroblasts repopulating the wound area is highly correlated with the pattern of the lamellae, suggesting contact guidance of intrastromal cell migration (Petroll et al., 2015b). Recently, a detailed temporal and spatial assessment of the changes in cell-ECM interactions and patterning during wound healing after PRK was performed, by using a combination of high resolution 3-D imaging techniques in a rabbit model (Kivanany et al., 2018a). Cell and lamellar patterning was assessed during all four phases of wound healing (migration, fibrosis, remodeling and regeneration), using multiphoton fluorescence and second harmonic generation (SHG) imaging. Under the wound bed, migrating keratocytes were highly aligned with the collagen lamellae as they repopulated the tissue, similar to freeze injury. However, a fibrotic healing response developed on top of the stroma, which was characterized by significant corneal haze, myofibroblasts, and fibronectin deposition that was organized randomly. Within this fibrotic tissue, collagen fibers have been shown to be coaligned with intracellular stress fibers, suggesting that cell-secreted collagen is organized via cell-matrix mechanical interactions (Farid et al., 2008). Over time, corneal transparency and thickness were restored, and collagen in the *fibrotic* region was *remodeled* into a more normal, interwoven pattern, with restoration of keratin sulfate labeling. In the native stromal wound bed, cells and ECM were co-aligned, and stromal re-growth (*regeneration*) came primarily from this area. Overall, F-actin and lamellar patterning in the *regenerative and remodeling* regions of healing suggest that cell/ECM mechanical interactions likely play a role in aligned collagen deposition and organization (Figure 9) (Kivanany et al., 2018a). Thus collagen lamellae appear to provide a template for fibroblast patterning during the migration, regeneration and remodeling phases of corneal wound healing.

Previous studies have demonstrated that UV cross-linking, which is increasingly used to treat keratoconus worldwide, more than doubles stromal stiffness in the rabbit cornea, and also produces significant alterations in lamellar organization and microstructure (Beshtawi et al., 2013; Bueno et al., 2011; Stewart et al., 2011; Tan et al., 2013; Wollensak and Iomdina, 2009). Interestingly, following CXL, which kills cells in the anterior stroma, the process of stromal repopulation can take over 3 months, suggesting that these structural/mechanical changes may inhibit normal cell migration (Mazzotta et al., 2015).

4.2. Regulation of cell patterning and differentiation by ECM composition

It is now well acknowledged that the composition of the local extracellular matrix (ECM) of the cornea stroma has a profound impact on keratocyte behavior. In the normal healthy corneal stroma, the most abundant ECM protein is type I collagen. However there are also significant amounts of type V and type VI collagen, as well as a variety of glycosaminoglycans and proteoglycans, such as keratocan, lumican, and decorin, that are interspersed among the aligned collagen fibrils (Zimmermann et al., 1986; Nakayasu et al., 1986). During wound healing and inflammation, small amounts of type III collagen are present as well (Massoudi et al., 2016)(Cintron et al., 1988). In addition to providing topographical cues, type I fibrillar collagen has binding sites for interactions with numerous other molecules (Di Lullo et al., 2002) such as multidomain adhesive glycoproteins (e.g. fibronectin, tenascin), glycosaminoglycans (e.g. keratan sulfate and hyaluronan), and both keratan sulfate proteoglycans (e.g. lumican, keratocan) and chondroitin sulfate proteoglycans (e.g. decorin). The binding of these molecules modifies the local ECM composition, and plays an important role for mediating keratocyte behavior. For example, the glycoproteins fibronectin (Erat et al., 2009) and tenascin (Midwood and Orend, 2009) both have been reported to bind to fibrillar collagen, and are present in the corneal stroma following injury. Fibronectin has been reported to enhance corneal fibroblast migration (Schmidinger et al., 2003), while tenascin reduces adhesion and migration (Schmidinger et al., 2003). Recent studies have suggested that proteoglycans also may modulate cell migration, contribute to differentiation (Kao and Liu, 2003), and bind certain growth factors (Massoudi et al., 2016; Schultz and Wysocki, 2008; Macri et al., 2007). For example, lumican deficiency has been reported to affect keratocyte proliferation and apoptosis during corneal stromal wound healing (Vij et al., 2004), and to effect TGF β signaling by binding to TGF β receptor 1 (TGF β R1) (Yamanaka et al., 2013). Likewise decorin has been shown not only to bind to type I collagen, but also to bind and inhibit all three isoforms of TGF β (Ferdous et al., 2007).

We previously demonstrated that ECM composition can modulate corneal behavior in 3-D culture in response to PDGF, which stimulates corneal fibroblast spreading and migration (Andersen and Ehlers, 1998; Andresen et al., 1997; Gallego-Munoz et al., 2018; Lakshman and Petroll, 2012; Petroll et al., 2008). Specifically, whereas corneal fibroblasts generally move independently within 3-D collagen matrices, fibrin induces a switch to an interconnected, *collective* mode of cell spreading and migration that is independent of differences in ECM stiffness (Miron-Mendoza et al., 2015; Miron-Mendoza et al., 2012). Furthermore, leading edge cells secrete and organize fibronectin into tracks during migration into 3-D fibrin matrices. These fibronectin tracks are used as conduits by the cells behind

them, which leads to the development of an interconnected cell/fibronectin network (Figure 10) (Miron-Mendoza et al., 2017). Interfering with cell attachment to fibronectin using an RGD peptide, anti-fibronectin antibody or $\alpha 5\beta 1$ antibody inhibits cell spreading and migration into fibrin. Interestingly, networks of interconnected fibroblasts are also observed during *in vivo* corneal wound healing (Jester et al., 1995; Kivanany et al., 2016; Kivanany et al., 2018a; Petroll et al., 1993; Petroll et al., 2015a), and it is hypothesized that these networks may facilitate or enhance wound repopulation and contraction.

5. Conclusions and future directions

Mechanical interactions between corneal keratocytes and the stromal ECM are regulated not only by growth factors and other cytokines, but also by biophysical signals due changes in ECM structure and mechanics. Signaling pathways that depend on the activity of Rho GTPases appear to play a central role in integrating these biochemical and biomechanical cues. Together these signals modulate the mechanical phenotype of corneal keratocytes and determine the pattern and amount of cellular force generation and ECM remodeling produced following injury or refractive surgery. Feedback between these biochemical and biophysical signals can also modulate keratocyte differentiation, myofibroblast transformation, and matrix synthesis and degradation.

As discussed in this review, most studies investigating the role of ECM stiffness, topography, and protein composition focus on one or two of these biophysical cues at a time. Often these studies incorporate the effects of one or more growth factors, but do not fully capture the multiplicity of biochemical and biophysical cues that keratocytes experience simultaneously *in vivo*. New sophisticated, multi-factorial culture platforms, which can modulate each of these cues independently, will be needed to disentangle the relationships between the biochemical and biophysical signals that regulate keratocyte behavior. This is currently not possible using existing organ culture or *in vivo* model systems. Understanding how corneal keratocytes integrate different combinations of cues will be critical to developing novel therapies to reduce or reverse corneal scarring, and to guide tissue engineering approaches to develop corneal stromal tissue replacements.

In addition, most studies of cell biomechanics focus on the activity of the F-actin cytoskeleton. However, the intermediate filament vimentin has also been associated with the myofibroblast transformation of corneal keratocytes, as well as the development of fibrosis *in vivo* (Bargagna-Mohan et al., 2015; Bargagna-Mohan et al., 2012; Chaurasia et al., 2009; Das et al., 2014). Moreover, numerous studies in other systems suggest that vimentin plays a central role in regulating key aspects of cell mechanical behavior, including mechano-sensing, polarization, and directional migration (Bargagna-Mohan et al., 2015; Costigliola et al., 2017; Ding et al., 2020; Gan et al., 2016; Mendez et al., 2014; Murray et al., 2014; Patteson et al., 2019). However, the role of vimentin in regulating the mechanical behavior of corneal keratocytes has not been established, and studies using other cell types have relied primarily on 2-D culture methods. It is also unclear how the activity of vimentin and other intermediate filaments interact with the Rho and Hippo signaling pathways, which have also been shown to modulate the myofibroblast transformation of corneal keratocytes. It is likely that these cytoskeletal proteins work cooperatively with classical F-actin driven mechanisms

to regulate ECM remodeling, subcellular patterns of force generation, as well as cell migration and patterning in the corneal stroma.

In summary, keratocyte mechanical behavior underlies many aspects of corneal disease, injury or surgical responses. Following injuries or incisional surgeries that penetrate the stroma, cellular force generation is needed to facilitate wound closure and restore the mechanical integrity of the cornea. However, following keratectomy or UV cross-linking procedures, the development of excessive force during wound healing can disrupt the normal lamellar architecture and reduce stromal transparency. Importantly, changes in tissue stiffness often accompany wound repair in the cornea, and myofibroblast transformation of corneal keratocytes is regulated, in part, by ECM stiffness. Thus, mechanical signaling pathways may be ideal targets for modulating the degree of corneal fibrosis produced after an injury. Mechanosensing pathways may also mediate keratocyte responses to diseases such as keratoconus (which decreases stromal stiffness) or treatments such as UV cross-linking (which increases stromal stiffness). Finally, the unique lamellar arrangement of collagen fibrils in the stroma provides strong topographical cues that can modulate keratocyte behavior. Specifically, cell patterning during intrastromal migration of corneal keratocytes is highly correlated with the pattern of the lamellae, suggesting contact guidance of keratocyte migration. Furthermore, following PRK surgery, collagen lamellae appear to provide a template for fibroblast patterning during both stromal tissue regeneration, and fibrotic tissue remodeling – two key events in the restoration of corneal transparency and thickness that are also important in corneal tissue engineering. Overall, a better understanding of keratocyte mechanobiology should have broad impact in both clinical and research settings.

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This review highlights how ECM structure and mechanics regulate the behavior of corneal keratocytes.

Changes in ECM stiffness regulate myofibroblastic transformation of keratocytes in 2D and 3D culture.

Topographical cues created via photolithography, electrospinning, and microfluidic deposition can modulate the patterning and differentiation of cultured keratocytes.

Rho GTPase-activity often coordinates the response of keratocytes to different biochemical and biomechanical cues.

Figure 1.

FEM strain maps generated using ANSYS, showing regions of matrix tension and compression surrounding a human corneal fibroblast expressing GFP- α -actinin within a 3-D collagen matrix. **A.** Cell-induced ECM deformation is observed following culture in media containing 10% fetal bovine serum (S+). Note that the ECM is under tension at the end of the cell, and under compression at the base of the pseudopodial process. **B.** The magnitudes of these deformations are reduced when the cell is switched to Y-27632 (10 μ M). **C.** Strain on the matrix is reestablished after switching back to S+. Strain is shown relative to the “relaxed” matrix configuration determined by treating cells with Cytochalasin D and TritonX-100. Bar on right shows scale for color contour strain maps in dimensionless units L/L. From (Vishwanath et al., 2003)

Figure 2. Corneal fibroblast spreading in response PDGF, which activates Rac. **A–C.** Cell-induced displacement and realignment of collagen fibrils was observed during PDGF-induced spreading. Tracking of the ECM displacements showed minimal collagen displacement prior to the addition of PDGF (B, red tracks, crosses mark position at time 0:00). However, following addition of PDGF, the matrix in front of the cell was pulled inward by the extending pseudopodial processes (C), resulting in compression of the ECM (yellow arrows). From (Petroll et al., 2008)

Figure 3.

Maximum intensity projections of fluorescently labeled f-actin (green) and confocal reflection images of collagen fibrils (red) near the interface between the inner and outer matrices of nested constructs. **A)** Following culture in 10% fetal bovine serum, migrating cells developed a bipolar morphology with occasional stress fibers along the cell body. Collagen fibrils were compacted and aligned parallel to the long axis of pseudopodia. **B)** Following culture in TGFβ1 (10 ng/ml), cells developed a broad morphology and intracellular stress fibers were observed. Collagen fibrils were compacted both around and

between the cells. C) Migrating cells in PDGF BB (50 ng/ml) were more elongated and had branching processes. Collagen fibrils remained more randomly aligned around the cells. From (Kim et al., 2010)

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Figure 4. Substratum stiffness modulates the mechanical phenotype of corneal keratocytes in the presence of TGF-β1. Representative traction stress maps of keratocytes cultured on either (A) 1 kPa or (B) 10 kPa polyacrylamide gels. Adapted from (Maruri et al., In Press).

Figure 5.

Fibroblast response to ECM compression using small microneedle. A-D. Human corneal fibroblast following 2 days of culture in media containing 10% fetal bovine, inside a 3-D collagen matrix. Needle was inserted axially into the matrix above the cell (A) without inducing changes in cell behavior (B). Pushing on the ECM towards the leading edge of a cell induced rapid cellular contraction and ECM compression along the cell body (C, arrows). This initial contraction was followed by re-spreading and tractional force generation (D, red tracks; crosses mark position immediately after needle push). E-H. Fibroblast response to ECM compression adjacent to cell body. Human corneal fibroblast 1 day after plating inside collagen matrix. Pushing small microneedles toward the side of the

cell had no significant effect on cell morphology or tractional force generation. From (Petroll and Ma, 2008)

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Figure 6. Effect of anisotropically ordered grooves and ridges on the orientation of primary rabbit corneal keratocytes, fibroblasts and myofibroblasts cultured on collagen-coated surfaces with (A-C) planar surfaces; (D-F) a 400-nm pitch size pattern; (G-I) a 1200-nm pitch size pattern; and (J-L) a 4000-nm pitch size pattern. Double-headed arrows indicate the direction of grooves and ridges on the patterned surfaces. Keratocytes were cultured in serum-free DMEM, and 10% FBS and 10 ng/mL TGF- β 1 were added to the culture medium for induction of the fibroblast and myofibroblast phenotype, respectively. Intracellular actin fibers were labeled with rhodamine-conjugated phalloidin. Scale bars, 100 μ m. From (Pot et al., 2010), with permission.

Figure 7.

The effect of electrospun fiber alignment on the behavior of human corneal stromal stem cells (hCSCs). Scanning electron micrographs of (a) aligned electrospun poly(ester urethane) urea (PEUU) fibers; (d) random PEUU fibers; and (g) PEUU cast film. Confocal laser-scanning micrographs of hCSCs on (b, c) aligned fibers, (e, f) random fibers, and (g, h) planar films at day 1 (b, e, h) and day 3 (c, f, i). Fluorescent labeling was with the cell viability marker Calcein AM. Fibril diameter = 165 ± 55 nm. From (Wu et al., 2012), with permission.

Figure 8.

Cell patterning during wound repopulation following freeze injury on aligned collagen substrates under different culture conditions. Each panel is a montage of F-actin images collected four days after injury. “W” marks the center of the original wound area, and red lines mark the approximate location of the original wound edge. The aligned collagen fibrils are patterned vertically. The concentration of PDGF BB was 50 ng/ml and the concentration of TGFβ1 was 5 ng/ml. From (Kivanany et al., 2018b)

Figure 9.

Anterior stroma in the regenerative region of the stroma 90 days after PRK, showing overlay of native collagen labeled with DTAF (green), forward scatter SHG of lamellae (blue), and cellular F-actin labeling (red). Note the high correlation in alignment of cells and both native and newly formed collagen lamellae. From (Kivanany et al., 2018a)

Figure 10.

Fibroblasts migrating through fibrin create fibronectin tracks. Nested matrix constructs were cultured for 48h in PDGF-containing media to stimulate cell migration. Corneal fibroblasts form an interconnected network as they migrate into the outer fibrin matrix (top part of image, white line delineates edge of inner matrix). This network contained significant fibronectin. As shown by arrows, fibronectin “tracks” were sometimes present in between cells. Scale bar is 100 μm . From (Miron-Mendoza et al., 2017)

Table 1

Topography	Dimensions	Substrate	Keratocyte source	Coating	Media	Ref
Ridges and grooves	RW = 70–2000 nm GW = 400–4000 nm GD = 600 nm	Silicon	Human	none	DMEM with 10% FBS	(Teixeira et al., 2004)
Ridges and grooves	RW = 2 μ m GW = 10 μ m GD = 30 μ m	PDMS	Human	Rat tail type I collagen	DMEM/HAM-F12 with 10% calf serum	(Vrana et al., 2008)
Ridges and grooves	Pitch = 400–4000 nm GD = 300nm	polyurethane	Rabbit	Collagen type I and III	DMEM	(Pot et al., 2010)
Ridges and grooves	Width = 350 nm - 10 μ m Pitch = 700 nm - 20 μ m Depth = 350 nm	PDMS Chitosan	Human	none	DMEM/HAM-F12 with 10% fetal calf serum	(Koo et al., 2011)
Grooves	Spacing = 200–300 nm Pore diameter = 0.45 μ m	PC transwell filter	HCSSCs	None	EMEM with/without 10% FBS	(Karamichos et al., 2014)
Random & Aligned fibers	$D_{\text{random}} = 956 \pm 56$ nm $D_{\text{aligned}} = 856 \pm 42$ nm	Gelatin/PLLA	rabbit	none	DMEM/F12	(Yan et al., 2012)
Random & Aligned fibers	$D_{\text{PCL}} = 108 - 536$ nm $D_{\text{PCL/PGS}} = 338 - 477$ nm $D_{\text{PCL/Chitosan}} = 108 - 144$ nm	PCL PCL/PGS PCL/Chitosan	Human keratocyte cell line	None	HAM-F-12/ Medium 199 with 5% FBS	(Stafiej et al., 2017)
Random & Aligned fibers	Diameter 165 ± 55 nm	PEUU	HCSSCs	None	Advanced DMEM with 10 ng/ml FGF	(Wu et al., 2013)
Random & Aligned fibrils	Not reported	Glass coverslips	Rabbit	Type I collagen fibrils	DMEM with PDGF, TGFB, or serum-free	(Kivanany et al., 2018b; Lam et al., 2019)

DMEM = Dulbecco's modified Eagle medium, FBS = fetal bovine serum, RW = ridge width, GW = groove width, GD = groove dept, PDMS = polydimethylsiloxane, PEUU = poly(ester urethane) urea, HCSSCs = human corneal stromal stem cells, FGF = basic fibroblast growth factor, PLLA = poly-L-lactic acid, PCL = polycaprolactone, D = diameter, PC = polycarbonate

EMEM = Eagle's Minimum Essential Medium