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Review

Biomechanical relationships between the corneal endothelium and Descemet's membrane

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ABSTRACT

The posterior face of the cornea consists of the corneal endothelium, a monolayer of cuboidal cells that secrete and attach to Descemet's membrane, an exaggerated basement membrane. Dysfunction of the endothelium compromises the barrier and pump functions of this layer that maintain corneal deturgesence. A large number of corneal endothelial dystrophies feature irregularities in Descemet's membrane, suggesting that cells create and respond to the biophysical signals offered by their underlying matrix. This review provides an overview of the bidirectional relationship between Descemet's membrane and the corneal endothelium. Several experimental methods have characterized a richly topographic and compliant biophysical microenvironment presented by the posterior surface of Descemet's membrane, as well as the ultrastructure and composition of the membrane as it builds during a lifetime. We highlight the signaling pathways involved in the mechanotransduction of biophysical cues that influence cell behavior. We present the specific example of Fuchs' corneal endothelial dystrophy as a condition in which a dysregulated Descemet's membrane may influence the progression of disease. Finally, we discuss some disease models and regenerative strategies that may facilitate improved treatments for corneal dystrophies.

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Contents

Abbreviations: CEnC, corneal endothelial cells; ECM, extracellular matrix; DM, Descemet's membrane; FCED, Fuchs' corneal endothelial dystrophy; AFM, atomic force microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; ABL, anterior banded layer; PNBL, posterior non-banded layer; TGF-β, transforming growth factor β; Wnt, Wingless/int; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; EMT, epithelial-mesenchymal transition; PI3K, Phosphoinositide 3-kinase; FGF-2, fibroblastic growth factor-2; PCL, posterior collagenous layer; ICE, Iridocorneal endothelial syndrome.

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The endothelium is the most posterior layer of the cornea and plays a critical role in maintaining corneal transparency, regulating deturgescence by providing both barrier and transport functions (Srinivas, 2010). A monolayer of corneal endothelial cells (CEnCs) maintains a barrier through tight junctions and adherens junctions (Srinivas, 2010; Ramachandran and Srinivas, 2010; Hartsock and Nelson, 2008; Noske et al., 1994). Osmotic pressure drives water from the anterior segment into the corneal tissues, and the endothelial layer maintains deturgescence through active fluid transport, energetically maintained by Na^{+}/K^{+}-ATPases (Bonanno, 2012; Fischbarg, 2003; Hatou et al., 2009). Corneal endothelial cells also produce a specialized basement membrane known as Descemet's membrane (DM) (Kabosova et al., 2007). Anterior to DM is the stroma, which constitutes the bulk of the cornea (Reinstein et al., 2009). Bowman's layer (Gordon et al., 1994), a specialized acellular extracellular matrix (ECM), separates the stroma from the anterior corneal epithelium in humans, but is absent in all domestic animals (Adler and Hart, 1992). A stratified squamous nonkeratinized epithelium makes up the anterior face of the cornea, with basal columnar cells that are anchored to the underlying anterior basement membrane (Adler and Hart, 1992).

It is well-documented that biophysical cues, such as substratum topography and stiffness, intrinsic attributes of all extracellular matrices, profoundly modulate a host of fundamental cell behaviors (Hay, 1985; Hao et al., 2014; Ingber, 2003; Wang et al., 2009; Janmey and Miller, 2011; Thomasy et al., 2012; Myrna et al., 2012; Raghunathan et al., 2013a; Dreier et al., 2013). Corneal cells interact with a rich variety of *in vivo* biophysical stimuli: the stroma and basement membranes present them with a range of stiffnesses and complex topographies (Abrams et al., 2000a). Our laboratory and others have documented that the biophysical attributes of matrices represent ubiquitous and potent cellular stimuli that modulate morphology (Petroll et al., 2004; Karamichos et al., 2007; McKee et al., 2011a; Raghunathan et al., 2013b; Koo et al., 2014), adhesion (Karuri et al., 2004), motility (Dreier et al., 2012; Raghunathan et al., 2013c), proliferation (Muhammad et al., 2015), gene expression and regulation (Raghunathan et al., 2014), and cell differentiation (Myrna et al., 2012; Dreier et al., 2013; Petroll and Lakshman, 2015) in a wide array of cell types. These cues also impact how cells respond to soluble signaling molecules and therapeutic agents (Thomasy et al., 2012; Myrna et al., 2012; Dreier et al., 2013). Insights gleaned from research on biophysical stimuli in the cornea inform potential therapies for conditions including corneal wounds (Okumura et al., 2015a; Petroll and Miron-Mendoza, 2015; Gao et al., 2015), as well as to tissue-engineered corneal constructs for transplantation (Shah et al., 2008). In particular, topographical and mechanical stimuli have been introduced to CEnCs to increase proliferation (Koo et al., 2014; Muhammad et al., 2015), maintain phenotype (Palchesko et al., 2015), and produce cell sheets for transplantation (Teichmann et al., 2013; Niu et al., 2014; Teo et al., 2012). Given the evidence supporting the role of mechanical signals in the function of CEnCs, it is surprising that the roles of these signals in homeostasis and pathogenesis have not been explored more thoroughly.

In this review, we present evidence that biophysical interactions must be considered when developing a complete model of the corneal endothelium in health and disease. We begin by describing experimental methods used to explore the biophysical microenvironment of corneal cells, particularly the stiffnesses of the tissues with which these cells interact. Next, we describe the ultrastructure of DM and the variations in the ECM at different stages of an organism's lifespan. We then highlight the signaling pathways that are likely to be involved in transducing mechanical signals from the ECM to the nucleus, thereby influencing cell behavior. To illustrate the reciprocal relationship between ECM and CEnCs, we describe a proposed interaction between DM and endothelial cells, engaging biophysical stimuli in Fuchs' corneal endothelial dystrophy (FCED), a corneal endothelial disease marked by characteristic abnormalities of DM. We then highlight the existing literature on *in vitro* studies of ECM biomolecules produced by CEnCs. We conclude by discussing corneal endothelial regeneration, an active area of research in which a deeper understanding of mechanical cues could have a beneficial impact.

1. Methods for characterizing the biophysical properties of corneal tissues

To investigate biophysical cues and their impact, it is necessary to characterize the mechanical microenvironment that cells experience *in vivo*, and to represent these cues *in vitro*. Tissues are mechanically quantified by measuring the elastic or Young's modulus (Askeland et al., 2011), a property that defines the sample's stiffness or its ability to resist deformation under an applied stress (Askeland et al., 2011). The elastic moduli of many biological tissues including the cornea have been reported and, interestingly, the reported values for a single tissue type can span several orders of magnitude, largely depending on the method of sample preparation and/or measurement (McKee et al., 2011b) (Table 1). Tensile measurements tend to be of higher magnitude than indentation-based measurements, such as those acquired through atomic force microscopy (AFM) or mechanical interferometry imaging, as the former measures bulk deformations in the tissue (with contributions from the ECM, cells, fibrillar and network-like proteins, and constrained water (McKee et al., 2011b)) whereas the latter methods measure localized deformations on small length scales (McKee et al., 2011b; Yoo et al., 2011).

In thin, heterogeneous tissue samples such as the endothelium or DM, AFM is ideal for measuring the micron-scale deformations that cells and their local ECM environments experience (McKee et al., 2011b; Last et al., 2010). Our lab has extensively used AFM to characterize the stiffness of the distinct layers of the human and rabbit cornea (Last et al., 2009, 2012; Thomasy et al., 2014) (detailed in Table 2), as well as the normal human trabecular meshwork (4.0 ± 2.2 kPa (Last et al., 2011)). The properties of the ECM can vary considerably between species (Thomasy et al., 2014; Worthington et al., 2014; Danielsen, 2004) (Table 1), and each layer in the rabbit eye is consistently softer than the corresponding structure in the human eye (Thomasy et al., 2014). For further information, we direct the reader to these reviews on the nuances of stiffness measurements in ocular tissues (McKee et al., 2011b; Last et al.,

Table 1
A comparison of measurements of the stiffness of Descemet's membrane in various species using several different analytical methods. In the mechanical interferometry imaging data set, the "short term" value refers to an instantaneous measurement whereas the "long term" values refers to a time dependent measurement including non-linear effects in response to constant stress over a 200 s interval. Atomic force microscopy measurements were instantaneous. Measured values are highly dependent on analytical methods, making it necessary to consider the strengths and limitations of each method during experimental design and data interpretation.

Species	Modulus	Measurement technique
Rat	2.81 ± 0.51 MPa (Danielsen, 2004)	Tensile testing
Cow	6.14 ± 0.41 MPa (Danielsen, 2004)	Tensile testing
Pig	4.29 ± 0.35 MPa (Danielsen, 2004)	Tensile testing
Human	2.57 ± 0.37 MPa (Danielsen, 2004)	Tensile testing
	50 ± 17.8 kPa (Last et al., 2009)	Atomic Force Microscopy
	1.8 ± 0.8 MPa (hydrated) and 4.8 ± 1.2 GPa (dehydrated) (Xia et al., 2016)	
	339.2 ± 22.2 kPa (short term) and 20.2 ± 0.71 kPa (long term) (Yoo et al., 2011)	Mechanical Interferometry Imaging
Rabbit	11.7 ± 7.4 kPa (Thomasy et al., 2014)	Atomic Force Microscopy

Table 2
Elastic moduli of corneal layers as measured by atomic force microscopy in humans and rabbits.

Corneal layer	Elastic Modulus	
	Human	Rabbit
Anterior Basement Membrane	7.5 ± 4.2 kPa (Last et al., 2009)	4.5 ± 1.2 kPa (Thomasy et al., 2014)
Bowman's Layer	109.8 ± 13.2 kPa (Last et al., 2012)	Absent
Stroma	33.1 ± 6.1 kPa (Last et al., 2012)	1.1 ± 0.6 kPa (anterior face) 0.38 ± 0.22 kPa (posterior face) (Thomasy et al., 2014)
Descemet's membrane	50 ± 17.8 kPa (Last et al., 2009) 1.8 ± 0.8 MPa (hydrated) and 4.8 ± 1.2 GPa (dehydrated) (Xia et al., 2016)	11.7 ± 7.4 kPa (Thomasy et al., 2014)
Endothelium		4.1 ± 1.7 kPa (Thomasy et al., 2014)

2010) and comprehensive summaries of biomechanical measurements of ocular tissue (Hugar and Ivanisevic, 2013; Raghunathan et al., 2016; Lombardo et al., 2012; Seifert et al., 2014; Dias and Ziebarth, 2013; Xia et al., 2016).

Topographical characterization of human (Abrams et al., 2000a) and canine (Abrams et al., 2002) DMs using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and AFM has documented DM to possess a rich felt-like surface of intertwining fibers, interspersed with elevations and pores. These features, shown in Fig. 1 (Abrams et al., 2002; Bentley et al., 2001), have sizes on the order of tens of nanometers and fractal dimension of ~2.2 (Abrams et al., 2000a). In comparison to the anterior corneal epithelial basement membrane, DM has smaller and more tightly organized features (Abrams et al., 2000a; Abrams et al., 2000b, 2002; Torricelli et al., 2013). The more compact organization of DM is reflected in its increased stiffness relative to the anterior corneal epithelial basement membrane (Last et al., 2009; Thomasy et al., 2014). Cellular basement membrane assembly is influenced by cell-matrix interactions (Glentis et al., 2014) and the biophysical attributes of ECM represent important inputs.

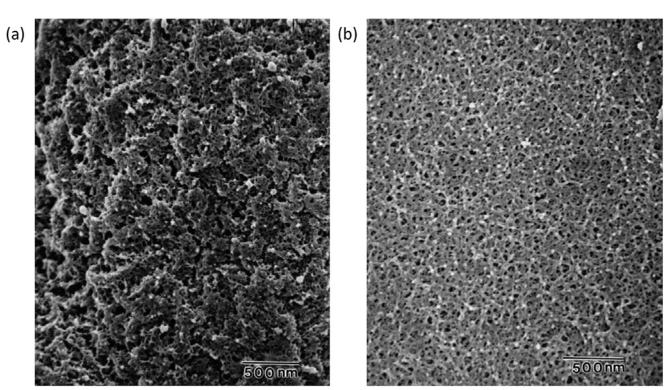

Fig. 1. The basement membranes of the canine cornea. (a) Scanning electron micrograph of the canine corneal epithelial basement membrane. The basement membrane has an intricate surface topography consisting of a meshwork of fibers and pores. (Reprinted with permission from Bentley et al, 2001. ©Association for Research in Vision and Ophthalmology) (b) Scanning electron micrograph of Descemet's membrane of the canine cornea. The complex topography of intertwined fibers and pores of varying sizes is similar to the topography found in the epithelial basement membrane. (Reprinted with permission from Abrams et al., 2002.).

2. The development and ultrastructure of Descemet's membrane

Like all basement membranes (LeBleu et al., 2007), DM is a compact sheet of ECM biomolecules, including proteins and glycosaminoglycans. The composition and ultrastructure of the deposited components vary by developmental stage and pathological state (Levy et al., 1996), forming distinct layers as they accumulate over a lifetime (Murphy et al., 1984a). Since the mechanical properties of ECM are related to its quantity, its constituent biomolecules, and its degree of crosslinking (through enzymes such as lysyl oxidase and transglutaminases) (Miller, 2016), we hypothesize that these variations would be reflected in differences in the biophysical cues experienced by endothelial cells on DM, as such influences have been observed in other cell types (Chaudhuri et al., 2014). Since there is no significant remodeling of DM after deposition, the transverse view serves as an interpretable historical record of development and pathology (Murphy et al., 1984a; Waring, 1982).

The thickness of human DM ranges from ~3 μm at birth to >10 μm in old age (Murphy et al., 1984a). The anterior region is deposited prenatally, from ~12 weeks after conception, to birth (Murphy et al., 1984a). By 16 weeks after conception, a striated pattern appears in transverse sections (Murphy et al., 1984a)and is known as the anterior banded layer (ABL), composed primarily of collagen IV and collagen VIII (Ljubimov et al., 1995; Sawada et al., 1990). Ultrastructurally in the ABL, collagen VIII forms several layers of stacked hexagonal lattices (Levy et al., 1996; Sawada et al., 1990), shown in Fig. 2 (Fratzl, 2008; Klintworth, 2009; Sawada, 1982). The lattice pattern arises from the molecular structure of collagen VIII as two types of short polypeptide chains, α1 and α2,

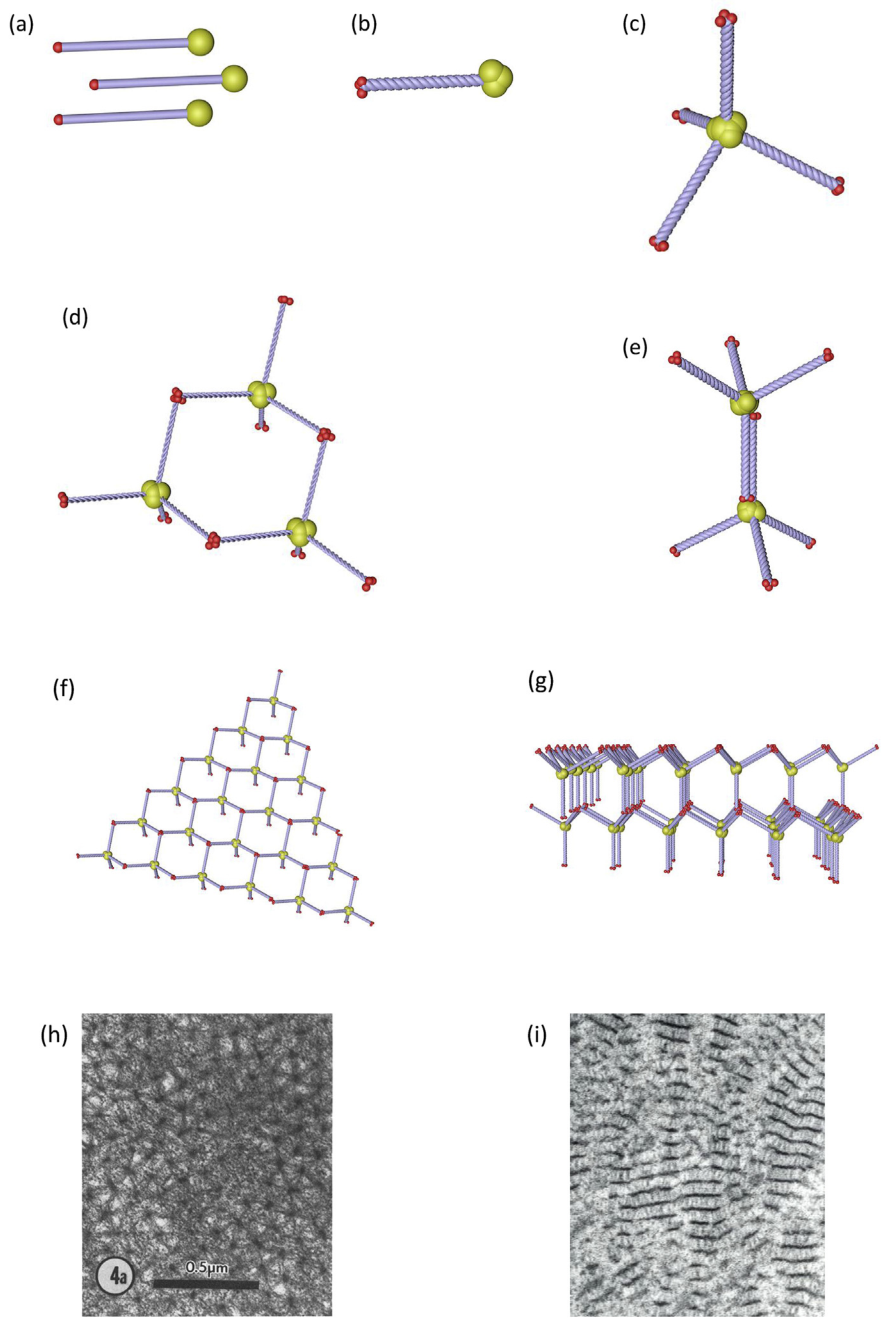

Fig. 2. The assembly of collagen VIII, an extracellular matrix protein critical in the assembly of Descemet's membrane. It comprises two types of polypeptide chains, α1(VIII) and α2(VIII) consisting of (a) two globular domains, the larger on the C-terminal side, connected by a rod-like triple-helical domain. (b) These polypeptides can form homotrimers. (c) Four of these homotrimers may associate through their C-terminal domains to form tetrahedral assemblies. These tetrahedra may assemble further via (d) N- to N- terminal interactions or (e) N- to C-terminal interactions. (f) Extended networks of collagen VIII assemblies may form a hexagonal network that (g) stacks vertically, shown here based on N-to N-terminal interactions. This theorized assembly pattern of collagen VIII corresponds to the ultrastructure of collagen VIII, imaged through transmission electron microscopy in Descemet's membrane. (h) In the en face section, collagen VIII appears as a hexagonal network, whereas (i) in the transverse section it appears as parallel bands. The imaged ultrastructure supports the theorized assembly of collagen VIII. ((h) reprinted with permission from Sawada, 1982. (i) reprinted with permission from Klintworth, 2009; licensed under the Creative Commons Attribution 2.0 Generic License).

assemble into homotrimers, then into a tetrahedral intermediate structure, and finally into hexagonal lattices (Stephan et al., 2004) which form characteristic bands (with 100 nm spacing) when stacked (Levy et al., 1996; Jakus, 1956). In normal human corneas, the two types of collagen VIII polypeptide chains are co-localized as bands within the ABL (Gottsch et al., 2005a). This characteristic banded pattern of the ABL is entirely absent in knock-out mutant mice that lack genes for both the α1 and α2 polypeptide chains (Hopfer et al., 2005). These mice have thinner DMs and a lower endothelial cell count suggesting the ABL to be critical to endothelial cell proliferation during development (Hopfer et al., 2005).

Near birth, endothelial cell secretion of collagen VIII diminishes as the cells transition from a proliferative to non-proliferative state, but collagen IV secretion continues (Kabosova et al., 2007; Hopfer et al., 2005; Kapoor et al., 1988). The ECM deposited thereafter lacks the banded pattern and is known as the posterior non-banded layer (PNBL) (Levy et al., 1996). The PNBL can be interspersed with small inclusions of fibrillar or banded ECM thought to arise from endothelial cells that transiently lose their postnatal differentiated state (Murphy et al., 1984a). Such anomalous deposits are often found within guttae and Hassel-Henle warts that form in aging corneas (Murphy et al., 1984a), suggesting that these abnormal structures form when endothelial cells undergo changes in ECM expression patterns over a lifetime. At this time, to the authors' knowledge, possible differences in the biophysical attributes of the ABL and PNBL remain unexplored. We feel it is likely that both compliance and/or topographic features would differ between these distinct regions of DM and that if such differences are present that they would influence the CEnC phenotype.

3. Biophysical signaling in the corneal endothelium

Since DM is the major source of biophysical stimuli for CEnCs, and since the cells themselves produce the ECM comprising DM, there is a bidirectional relationship between endothelial cells and the ECM they deposit (Waring, 1982; Zagorski and Naumann, 1992) (Fig. 3). Such a relationship, known as "dynamic reciprocity" (Bissell et al., 1982; Bornstein et al., 1982; Bissell and Aggeler, 1987), has been observed in many physiological systems, including the corneal stroma (Petroll and Miron-Mendoza, 2015). A detailed understanding of the signaling pathways that underlie these bidirectional relationships may reveal new targets for therapeutic intervention by interrupting the positive feedback loop of dysfunctional ECM – produced under pathological conditions – inducing further pathological cell behavior. The primary pathway impacted by the biophysical attributes of DM is the mechanotransduction pathway, which intersects downstream with the Hippo (Dupont et al., 2011), transforming growth factor β (TGF-β) (Edlund et al., 2002), Wingless/int (Wnt) (Akiyama and Kawasaki, 2006; Aragona et al., 2013), and other signaling pathways (Jahed et al., 2014) that control gene expression. The intersection of critical signaling pathways is presented in Fig. 4 (Morgan et al., 2013).

Mechanotransduction: Mechanotransduction is the process by which cells sense extracellular mechanical cues, including ECM stiffness, topography, and spatial constraints, and convert them into intracellular biochemical signals leading to cellular responses (Ingber, 2003; Wang et al., 2009). Transmembrane mechanoreceptors such as integrins and syndecans bind to ECM molecules and detect stresses in the extracellular environment (Vogel and Sheetz, 2006). On the cytoplasmic side, they are linked to the actin cytoskeleton which is critical in generating and transmitting tension throughout the cell, controlling cell morphology and activating downstream signaling pathways (Schwarz and Gardel, 2012). The cell can probe the stiffness of its surroundings by contracting its actin cytoskeleton – a compliant substrate offers less resistance to deformation and induces less stress in the contractile machinery than a stiff one (Schwarz and Gardel, 2012). Mechanical stresses transmitted through mechanoreceptors to the cytoskeleton can alter the assembly dynamics of actin filaments, inducing conformational changes in actin-binding proteins that play regulatory roles further downstream (Romet-Lemonne and Jegou, 2013). The cytoskeleton also links to structures in the nucleus, coupling integrin activation directly to nuclear conformational changes and gene transcription activity (Maniotis et al., 1997). It thus plays a central role in enabling extracellular biophysical cues to regulate a variety of cell behaviors, including survival, proliferation, differentiation, migration, adhesion, and polarity (Schwarz and Gardel, 2012; Choquet et al., 1997; Zimmermann and David, 1999; Ross et al., 2013). Integrins additionally regulate the actin cytoskeleton (Hall, 1998) by activating the Rho/ROCK pathway through Src-family kinases and Rho-family GTPases (Huveneers and Danen, 2009) and influence migration, proliferation, and apoptosis (Olson et al., 1995). Rho-associated protein kinase (ROCK) has been linked to proliferation (Okumura et al, 2009, 2011a, 2012, 2014a, b; Koizumi et al., 2012), wound healing processes (Okumura et al, 2011a, 2011b, 2013a, 2014a, b, 2015a) and the barrier function (Srinivas, 2010, 2012; Jalimarada et al., 2009; Rajashekhar et al., 2014) in CEnCs (Koizumi et al., 2012).

YAP/TAZ and Hippo: The Hippo pathway, critical for management of organ size during development, has been recently shown to intersect with the mechanotransduction pathway. Two transcriptional regulators and key players in the Hippo pathway, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), were identified by Dupont and colleagues as a necessary relay for conveying mechanotransduction signals to the nucleus for gene regulation (Dupont et al., 2011; Halder et al., 2012). In cells adherent to soft substrates or confined to a small area, YAP/TAZ accumulate in the cytoplasm and eventually degrade, whereas when cells are spread or attached to stiff surfaces, YAP/TAZ relocate to the nucleus and induce gene expression (Dupont et al., 2011; Dupont, 2015). The mechanisms behind these localization patterns are unclear, although the regulation of the actin cytoskeleton by the mechanotransduction pathway appears to play key roles (Dupont et al., 2011; Halder et al., 2012). For instance, G-protein coupled receptors acting through the actin cytoskeleton inhibit YAP localization in the nucleus by acting on LATS 1/2 (Yu et al., 2012). The specific roles of YAP/TAZ in the corneal endothelium are currently under investigation: YAP expression in the nucleus and cytoplasm increases in CEnCs at the periphery of the endothelium, coinciding with higher proliferative potential (Zhao

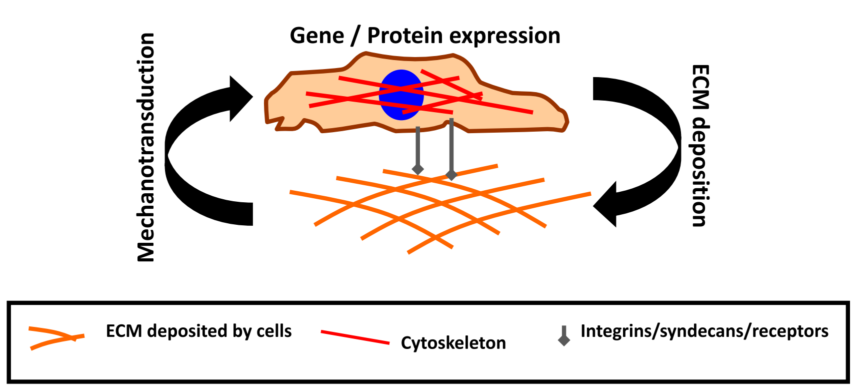

Fig. 3. Dynamic Reciprocity. Cells synthesize ECM proteins and deposit them into the extracellular space. The proteins assemble the matrix, presenting a rich set of topography and modulus cues to the cells. Integrins, syndecans, and other receptors mechanotransduce biophysical cues into the cells. Signaling molecules and the cytoskeleton convey these signals to the nucleus, where they influence cell behavior in many ways, including through changes in the expression of ECM genes and proteins.

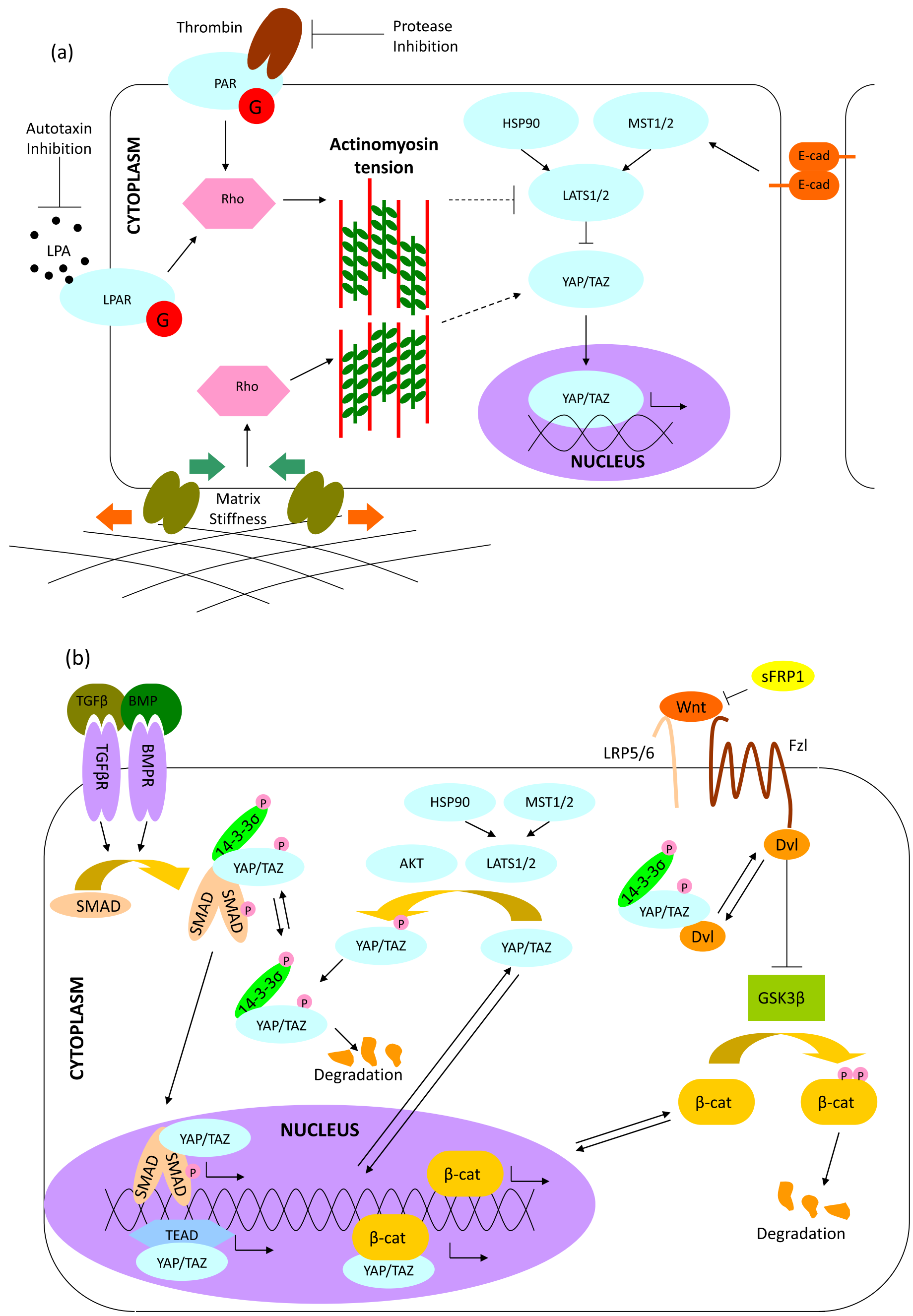

Fig. 4. Signaling pathways linked to mechanotransduction. (a) Mechanical regulation of the Hippo pathway. Hippo is regulated by multiple signals generated by the physical (matrix stiffness) and biochemical (e.g. LPA, thrombin) environment. Importantly, many of these signals are modulated by tension in the actinomyosin cytoskeleton. Modulation of cytoskeletal mechanics through G-protein coupled receptors and matrix biophysics can likewise inhibit YAP/TAZ directly at the nuclear translocation stage or through activation of Hippo components. Note: This schematic is simplified to clarify the major components in the mechanical regulation of YAP/TAZ signaling. (b) Crosstalk between YAP/TAZ and TGF-β, and between YAP/TAZ and Wnt. TGF-β superfamily signaling is initiated by the binding of an extracellular ligand (e.g. TGF-β and BMP), which leads to the phosphorylation of SMADs and the formation of a complex with a Co-SMAD and 14-3-3s. After translocation to the nucleus, these complexes initiate the TGF-β/BMP transcriptional program. The SMAD complex also interacts with YAP/TAZ to initiate different transcriptional programs in the nucleus. Canonical Wnt is initiated by the binding of a Wnt ligand to the Fzd/LRP receptor complex. This induces the inhibitory behavior of Dvl on the Axin/APC/GSK3b complex, freeing β-catenin to translocate to the nucleus and initiate the Wnt transcriptional program. YAP/TAZ can inhibit Wnt signaling through inhibition of Dvl in the cytoplasm (TAZ) or in the nucleus (YAP) or cytoplasmic sequestration of β-catenin (YAP). Alternatively, YAP can encourage the transcriptional activity of β-catenin. Note: This schematic is simplified to clarify the intersections of YAP/TAZ and Wnt signaling. (Adapted with permission from Morgan et al., 2013).

et al., 2015). Additionally, cultured CEnCs have been induced to proliferate by increasing YAP expression (Hsueh et al., 2015). Because YAP and TAZ are targets and effectors of numerous cell-signaling pathways, including Hippo, TGF-β, Akt, and both canonical and non-canonical Wnt pathways (Morgan et al., 2013; Park et al., 2015; Ye et al., 2012), they can orchestrate the influence of mechanical cues on a wide range of cell behaviors, including epithelial-mesenchymal transition (Diepenbruck et al., 2014).

TGF-β: Members of the TGF-β family are cytokines that are generally inactive in the ECM until released upon injury or mechanical stress, and then bind to membrane receptors (Piersma et al., 2015). This initiates downstream signaling, activating several types of Smad proteins that form a complex, translocate to the nucleus, and promote transcription (Piersma et al., 2015). In CEnCs and other cell types, they induce the synthesis, remodeling, and degradation of ECM proteins (Usui et al., 1998), and trigger the transdifferentiation activity known as epithelial-mesenchymal transition (EMT) (Roy et al., 2015; Zavadil and Bottinger, 2005; Verrecchia and Mauviel, 2002), which is a feature of FECD (Okumura et al, 2013b, 2015b; Matthaei et al., 2014; Matthaei et al., 2015). Changes in matrix protein expression are likely to alter the biophysical properties of the secreted ECM, invoking a mechanotransduction response within the cell. Additionally, the Smad protein complex interacts with YAP/TAZ in some cells to evoke an enhanced fibrotic or metastatic response (Piersma et al., 2015; Hiemer et al., 2014). Through these mechanisms, the TGF-β pathway may impact the dynamic reciprocity between CEnCs and the ECM.

AKT: The Akt pathway promotes survival and growth in response to extracellular signals, including cytokines, hormones, and ECM interactions. It is initiated when the cell surface receptor phosphatidylinositol 3-kinase (PI3K) is activated. Akt, a serine/threonine kinase, is then activated and initiates downstream processes (Osaki et al., 2004). In CEnCs, fibroblast growth factor 2 (FGF-2) initiates the Akt pathway and promotes proliferation (Lu et al., 2006). Furthermore, Akt-induced proliferation in CEnCs is inhibited by TGF-β2 via the latter's transcription products (Lu et al., 2006). The Akt pathway also intersects with the downstream relays of the mechanotransduction pathway: Akt interacts with YAP/TAZ and localizes them in the cytoplasm while limiting their degradation (Basu et al., 2003). Through these interactions, the Akt pathway has multiple impacts on dynamic reciprocity.

Wnt: The Wnt pathway plays key roles in development, homeostasis, disease, and mechanotransduction. In the canonical pathway, Wnt ligands bind to membrane receptors, initiating a downstream signaling cascade that stabilizes intracellular β-catenin, saving it from degradation and allowing it to regulate transcription in the nucleus (Piersma et al., 2015). If sufficient Wnt antagonists are present, the pathway is inhibited and cytoplasmic β-catenin is marked for degradation, limiting its transcriptional activity (Piersma et al., 2015). However, β-catenin can be rescued from degradation by association with YAP/TAZ. The Wnt pathway is thereby influenced by downstream relays of mechanotransduction. A few more interactions are worth noting: In the corneal endothelium, matrix metalloproteinase inhibitors can reverse EMT through inhibiting the Wnt pathway, thereby linking pathological cell behavior to ECM remodeling (Ho et al., 2015). A non-canonical (β-catenin independent) Wnt pathway regulates the cytoskeleton via Rho GTPases (Schlessinger et al., 2009). Rho GTPases activated through PI3K are linked to increased EMT in CEnCs (Lee and Kay, 2006). This EMT increase occurs when CEnCs are induced to proliferation through disruption of their junctions and the addition of FGF-2 (Lee and Kay, 2006). However, Wnt activation can be bypassed by silencing nuclear p150 catenin to activate the Rho pathway (Zhu et al., 2012). All these interactions provide points at which the Wnt pathway can have an influence on the dynamic reciprocity between the endothelium and DM.

The signaling pathways described above converge on two cellular responses in particular: proliferation and EMT-induced ECM expression. Changes in the ECM, in turn, influence the cell mechanically, creating a closed loop of influence in which abnormal matrix can trigger the expression of more abnormal matrix. Both cellular responses are relevant to the corneal endothelium in health and disease.

4. Dysfunction of the corneal endothelium

Primary corneal diseases are widespread and represent the third leading cause of blindness (Klintworth, 2009; Bourne, 2003). Diseases of the corneal endothelium are especially burdensome. While the CEnCs of some species, such as rabbits and cows, retain some proliferative capabilities throughout life (Joyce et al., 1996), human CEnCs are especially notable for their low proliferation rate, which is insufficient to replace cells lost through age, trauma, or disease (Murphy et al., 1984b). The remaining cells migrate and enlarge to maintain the intact monolayer (Hoppenreijs et al., 1992) leading to subsequent cellular polymegathism and pleomorphism in the endothelium (Matsuda et al., 1985). If the endothelial cells cannot maintain an intact monolayer, their barrier as well as pump functions are disrupted leading to stromal edema and a progressive decrease in visual acuity (Klintworth, 2009). The endothelium can degenerate because of primary corneal dystrophies, or secondary to other disturbances to the eye such as glaucoma (Gagnon et al., 1997), uveitis (Olsen, 1980), or intraocular surgery (Liesegang, 1991). The likelihood of secondary changes such as bullous keratopathy are increased if the endothelium has pre-existing signs of vulnerability, and candidates for anterior segment surgery should be screened for low endothelial cell counts (Irvine, 1956; Schultz et al., 1986; Ventura et al., 2001). The health of CEnCs is closely linked to the state of DM to which they adhere. Many dystrophies are associated with the production of abnormal ECM or amyloid deposits that are likely to change the topography and modulus of the membrane (Soumittra et al., 2014; Loganathan and Casey, 2014), suggesting that biophysical interactions with cells may have an impact on a wide range of corneal diseases. Here, we provide a brief introduction of the biophysical properties associated with corneal endothelial dystrophies.

4.1. Fuchs' corneal endothelial dystrophy

Fuchs' corneal endothelial dystrophy is the most common corneal endothelial dystrophy in the United States (Klintworth, 2009). Clinically, FCED manifests as a thickened and multi-layered DM, with the presence of corneal guttae, corneal edema and deterioration of vision (Gottsch et al., 2005a).

It occurs in two forms, a late-onset variant and a rarer early-onset variant (Hamill et al., 2013). In addition to their clinical presentation of thickened DM and gutta formation, the two forms of FCED involve Collagen VIII in distinct ways. Fuchs' dystrophy has been linked to mutations in genes with a variety of functions, begging the question of how a diverse set of genes lead to the common phenotype of abnormal ECM production. Here, we discuss the alterations of ultrastructure and composition of DM in FCED, discuss the associated genetic anomalies that may alter the ECM composition and organization, and consider how these changes may impact intrinsic biophysical attributes, cell function and survival.

Ultrastructural studies of DM in FCED patients reveal a characteristic pattern. Descemet's membrane of FCED patients' corneas is thicker than that of age-matched normal corneas, although the PNBL is thinner. In late-onset FCED, the ABL is slightly thicker than normal. There is a gradual transition from the PNBL to a posterior collagenous layer (PCL) interspersed with banded patterns termed "wide-spaced collagens" similar to that of the ABL (Levy et al., 1996; Waring, 1982; Bourne et al., 1982). Some corneas, particularly those with pronounced edema, also have a loose "fibrillar layer" between the banded PCL and endothelial cells (Bourne et al., 1982). The arrangement of wide-spaced, amorphous, and fibrillar ECM is increasingly irregular and distorted toward the posterior of the cornea (Bourne et al., 1982). The guttae that appear on the posterior DM in FCED are also composed of ECM similar to the PCL (Bourne et al., 1982). In early-onset FCED, the ABL is notably thicker than normal (Gottsch et al., 2005a). It is followed by a PNBL similar to that in late-onset FCED, and an internal collagenous layer (ICL) reminiscent of the PCL in late-onset FCED (Gottsch et al., 2005a). Posterior to this is a very thick, collagen VIII-rich posterior striated layer, which does not occur in late-onset FCED (Gottsch et al., 2005a). Guttae are smaller than in late-onset FCED and buried within other deposited ECM material (Gottsch et al., 2005b).

Corneal endothelial cells have the latent ability to synthesize a larger set of ECM protein types than they normally produce, and diseases may arise when regulation of protein expression is dysfunctional (Lee et al., 1994). In late-onset FCED, there is increased deposition of collagen IV, laminin, fibronectin, and several other ECM components in the posterior of DM (Gottsch et al., 2005a; Weller et al., 2014).

While Collagen VIII is normally found only in the ABL of DM, appearing as arrayed domains of the $\alpha 1$ and $\alpha 2$ chains (Gottsch et al., 2005a), this arrangement of Collagen VIII is disrupted in FCED. Collagen VIII is also expressed in the PCL, indicating that its production is resumed after cessation in infancy (Levy et al., 1996). Interestingly, Collagen VIII plays a different but central role in early-onset FCED also. This condition is associated with mutations in COL8A2. A few familial pedigrees and sporadic individuals have been identified that carry missense mutations in COL8A2 (either Q455K, Q455V, or L450W (Gottsch et al., 2005b; Biswas et al., 2001)). In patients with the L450W mutation, intracellular and extracellular collagen VIII accumulation correlated with the severity of the disease (Zhang et al., 2006). Corneas of patients with early-onset FCED, as well as corneas in mouse models with COL8A2 knock-in mutations, have a thickened DM, guttae, changes in endothelial cell morphology, and cell loss, supporting the idea that mutations in Collagen VIII can produce signs of FCED independently of other mutations (Jun et al., 2012; Meng et al., 2013).

It is as yet unclear what mechanisms trigger changes from one type of ECM composition to another, and why the various layers in normal and pathogenic DMs appear as they do. However, we can speculate on how these ECM alterations modify the biophysical environment experienced by cells. For instance, since point mutations can affect protein folding, it is likely that mutant COL8A2 in early-onset FCED leads to the expression of collagen VIII chains that do not assemble into the same macromolecular structures as normal chains do (Bateman et al., 2009). This could alter the biophysical properties of DM, thereby triggering expression changes within the cell.

In addition to the COL8A2 mutations in early-onset FCED, several genetic mutations have been associated with FCED, and these are comprehensively reviewed in the literature (Klintworth, 2003, 2009). The affected genes TCF4 and TCF8 express the transcription factors E2-2 and ZEB1 respectively, which are known to promote EMT (Chung et al., 2014; Baratz et al., 2010). TCF8 also alters the expression of Collagen IV, which is abundant in DM (Chang et al., 1996). Changes in these key functions of ZEB1 may contribute to abnormal ECM deposition. Several mutations, in COL8A2 (Zhang et al., 2006), SLC4A11 (Vilas et al., 2012), and LOXHD1 (Riazuddin et al., 2012), cause cytoplasmic accumulation of proteins. These may induce gutta formation through a mechanism involving the accumulation of proteins in the endoplasmic reticulum, and subsequent unfolded protein response, that has been proposed by Son et al. (Son et al., 2014).

In FCED, the PCL is less stiff than the PNBL of the normal DM, and this may be due to alterations in the composition, assembly patterns, and hydration content of the matrix (Xia et al., 2016; Thomasy et al., 2016a). The composition of the ECM can greatly influence the modulus (Black et al., 2008). The heterogeneity of the PCL ultrastructure may interrupt the packing structure of ECM biomolecules, changing the PCL density and modulus relative to the normal PNBL (Hwang and Brodsky, 2012; Hwang et al., 2010). As the DM is normally defined by the network structure of collagen IV, the presence of a fibrillar layer is also likely to change the surface attributes with which endothelial cells interact (Muiznieks and Keeley, 2013). Wide-spaced collagens also alter the topography of the DM surface, as revealed by AFM imaging (Xia et al., 2016).

As guttae are physical structures growing on DM, they exert particular biophysical cues on CECs. The guttae appear as round, flat-topped growths that are 5–50 μm in diameter (Xia et al., 2016) and up to 20 μm in height (Mehta et al., 2016). The height of topographic features has been shown to impact cell behaviors though at scales markedly smaller than guttae (Tocce et al., 2010). A preliminary report suggests that the micron-scale dimensions of guttae can impact CEnC behaviors including migratory behavior and monolayer formation *in vitro* (Zhang et al., 2006). In histopathological sections of FCED corneas, the cells exhibit a spread morphology over guttae with cell bodies displaced laterally to the guttae stems but with sections of cell membrane intact over gutta apices (Bergmanson et al., 1999). This displacement is sufficient to distort the cells and apply tensile forces to their actin cytoskeletons. Such mechanical stretch has been associated with EMT in other cell types (Heise et al., 2011), and this distortion of CEnCs may induce or contribute in part to the EMT-like behavior seen in FCED. With limited space for organelles in the stretched areas and for Na^+/K^+-ATPase on the diminished lateral membranes, the pump function of the corneal endothelium may be quite diminished (Bergmanson et al., 1999). However the barrier function of the endothelium is retained as cells remain adherent to one another until late in disease progression (Bergmanson et al., 1999).

For a complete understanding of the role of biophysical cues in pathogenesis of FCED, we need to fully characterize the biophysical cues presented by DM in FCED, understand how the signaling pathways initiated by mechanotransduction determine the downstream impact on cell health, gutta formation and ECM protein expression. While there are several animal models for FCED, including transgenic mice (Jun et al., 2012; Meng et al., 2013) and Boston terriers (Thomasy et al., 2016b), the biophysical attributes of DM and endothelium in these species remains uncharacterized. Given the challenges in observing the early development of FCED, *in vitro* cell models of the disease could help us fill these knowledge gaps.

4.2. Other endothelial disorders

Posterior polymorphous corneal dystrophy is a rare, slowly progressive or non-progressive condition that develops during the first decade of life but is often asymptomatic (Bozkurt et al., 2013). It is characterized by a multilamellar DM with vesicle- and band-like lesions and focal nodular excrescences (Klintworth, 2009; Henriquez et al., 1984). Congenital hereditary endothelial corneal dystrophy presents as a cloudiness or opacification and edema of the cornea that is present at birth or develops in infancy (Klintworth, 2009). Disruption of collagen fibrils occurs in the stroma as well as the production of a fibrous collagen layer posterior to DM (Klintworth, 2009). Subepithelial amyloid deposits have been reported in several cases (Klintworth, 2003; Mahmood and Teichmann, 2000; Vemuganti et al., 2002; Pandrowala et al., 2004; Al-Shehah et al., 2010). X-linked corneal endothelial dystrophy was recently discovered in 60 members of a single family (Schmid et al., 2006). The corneas developed opacifications including congenital cloudiness, subepithelial band keratopathy, and endothelial pits (Schmid et al., 2006). Examination of one cornea revealed an irregularly thickened DM, consisting of an abnormal ABL, an abnormal posterior banded zone of varying ultrastructure and thickness, and an absent PNBL (Schmid et al., 2006). Iridocorneal endothelial syndrome (ICE) is a group of related corneal disorders in which endothelial cells migrate onto the iris and block the drainage angle, leading to iris atrophy and glaucoma (Sacchetti et al., 2015). In a study of 27 ICE affected corneas, the majority possessed DMs with a posterior layer of microfibrils embedded in an amorphous matrix. In several others, there were no banded collagenous layers either anterior or posterior to the non-banded layer (Levy et al., 1995). The commonality in features across several corneas suggests these patterns are specific to the mechanisms of ICE (Levy et al., 1995).

Although the mechanical, topographical, and biochemical properties of DM in these diseases have not been well characterized, we note a recurring incidence of abnormal ultrastructure and the appearance of lesions, excrescences and craters in DM (Klintworth, 2009; Henriquez et al., 1984; Schmid et al., 2006; Sacchetti et al., 2015; Zhang and Patel, 2015). These features very likely offer abnormal topographical and mechanical cues to endothelial cells growing on and interacting with the membrane, which could trigger pathological processes such as EMT (Gjorevski et al., 2012) or apoptosis (Zhang et al., 2011). While it is yet unclear whether these biophysical abnormalities in DM are responsible for, or merely coincide with, endothelial cell dysfunction and premature degeneration, there are other instances in the biomedical literature of dysregulated extracellular matrix causing pathological behavior including fibrosis, atherosclerosis and tumorigenesis (Cox and Erler, 2011; Kothapalli et al., 2012). Furthermore, our lab has demonstrated that changes to substratum compliance and topography can promote the transformation of corneal fibroblasts to myofibroblasts (Myrna et al., 2012; Dreier et al., 2013), leading us to hypothesize that the biophysical characteristics of DM impact endothelial cell morphology and function.

5. *In vitro* studies of ECM in corneal endothelial cells

To delineate the mechanisms of corneal endothelial dystrophies and identify efficacious treatments, it is critical to establish *in vitro* disease models using cell culture. A complete description of mechanism needs to capture the bidirectional interactions between cells and ECM, and characterize ECM expression changes in the presence of mutations, stress, EMT, and disease states. While some studies of CECs have measured ECM gene and protein expression changes, the resulting matrix has rarely been characterized.

The major protein component of the ECM expressed by CEnCs is collagen (Tseng et al., 1981), predominantly collagens IV, VIII, XII, and XIII (Weller et al., 2014). The production of a matrix, similar to DM in appearance and collagen content, by rabbit CEnCs was first reported in 1974 (Perlman and Baum, 1974). Over the next two decades, studies showed that the content of the ECM produced by CEnCs could be modulated by treating the cell culture with growth factors, such as FGF-2 (Kay et al., 1993) and TGF-β (Usui et al., 1998), that also play a role in EMT (Lee et al., 2012). Cells cultured at suboptimal conditions, e.g. passaged repeatedly at subconfluence in the absence of FGF-2, eventually shift to a higher production of collagen I (Tseng et al., 1981). One study demonstrated that ECM production by CEnCs in response to TGF-β depends on the expression levels of the EMT-inducers Snail1 and ZEB1, which tend to be elevated in FCED-derived cells (Okumura et al., 2015b). Immortalized cells derived from normal and FCED-affected endothelia were cultured to deposit ECM on polyester membranes, and transmission electron microscopy was used to quantify and compare the thicknesses of the deposited matrices. In the FCED-derived cells, expression of collagen I, collagen IV, and fibronectin was elevated, and the ultrastructure revealed more fibrillar ECM compared to in normal phenotype cells.

A key *in vitro* strategy to understanding the role of ECM in disease and mechanotransduction is to culture cells on ECM substrates with differing biophysical attributes and investigate their responses. An early study culturing normal CEnCs for a week on FCED-derived DM did not show abnormalities in morphology or monolayer formation, although the study was limited in time and did not investigate cell function (Raphael et al., 1992). Interestingly, cells cultured on pseudophakic bullous keratopathy-derived membranes adopted an abnormal non-confluent stellate form, indicating that this rapid-onset condition disrupts DM and modulates cell growth more acutely (Raphael et al., 1992). CEnCs have also been plated on ECM-coated tissue culture dishes (Choi et al., 2013) and soft substrates (Palchesko et al., 2015) to assess which surfaces best promote the normal corneal endothelial phenotype. Synthetic substrates that are biomimetic of DM stiffness have also been developed, particularly to enhance proliferation and monolayer stability in artificial corneal grafts (Palchesko et al., 2015; Palchesko, et al., 2012). Another approach to study cell response to ECM is to deposit a matrix from cells in a long-term culture, decellularize the matrix, and then use the matrix as a substrate for a *de novo* cell culture (Castaldo et al., 2013) but this approach has not been applied to CEnCs to the authors' knowledge.

The development of *in vitro* models that mimic both homeostatic and disease states would better enable us to recapitulate the abnormal ECM signals in corneal dystrophies, assess their impact on cell fate, and characterize the resultant expressed matrix biomolecules.

6. Impact of biophysical cues on regenerative treatments

The successful *in vitro* culture of CEnCs is a prerequisite for novel regenerative treatments. Presently, the most common treatment for corneal endothelial dystrophies is transplantation of the full-thickness cornea or DM with associated CEnCs, but a global shortage of available donors has driven the development of cell replacement therapy approaches (Zavala et al., 2013) and synthetic corneal endothelium grafts (Zavala et al., 2013; Mimura et al., 2013). Cell replacement therapy involves the culture and expansion of CEnCs *in vitro*, and subsequent transplantation into the eye without a carrier material. Successful cultures have been established by overcoming proliferative arrest of CECs through the disruption of junctions (Senoo et al., 2000), and treatment with small molecules (e.g. Rho-kinase inhibitors) (Okumura et al., 2014a, b; Hsueh et al., 2015) and growth factors (e.g. FGF-2) (Tseng et al., 1981; Chen et al., 2001). Furthermore, corneal endothelial precursor cells from the peripheral cornea have a higher than average proliferative capacity and corneal endothelial stem cells are located in a sequestered niche at the limbus between the endothelium and trabecular meshwork (Whikehart et al., 2005). Cultures established with the endothelial stem cells have been delivered into animal models of bullous keratopathy, where they adhered to DM and enhanced corneal clarity (Mimura et al., 2005).

For ease of delivery, it may be necessary to culture corneal endothelial cells on a carrier before implanting the construct as an artificial graft. Grafts have been prepared by culturing endothelial cells on native tissue such as decellularized DMs (Bayyoud et al., 2012), amniotic membranes (Ishino et al., 2004), and lens capsules (Yoeruek et al., 2009). Carriers of cultured cells have also been synthesized with biological materials such as collagen I (Gruschwitz et al., 2010; Mimura et al., 2004), silk fibroin (Madden et al., 2011), and chitosan (Gao et al., 2008). ECM-based coatings are frequently applied to the carriers to enhance cell function (Choi et al., 2013; Desgranges et al., 1992; Underwood and Bennett, 1993; Blake et al., 1997). Blended biomaterials combine the desired properties of their components to produce surfaces that have clarity, biocompatibility, and mechanical strength (Wang et al., 2012; Chen et al., 2015). Another desired trait, the nondestructive detachment of intact endothelial monolayers, has been achieved through the development of thermoresponsive (Teichmann et al., 2013; Hsiue et al., 2006; Gotze et al., 2008) and biodegradable (Gao et al., 2008; Wang et al., 2012; Hsiue et al., 2006; Hadlock et al., 1999) substrates.

These artificial grafts and carriers have not yet recapitulated the functionality of the native cornea to a clinically-relevant degree: cell density and quality do not match the native endothelium, the Na^+/K^+-ATPase pump function is 75–95% that of normal human donor corneas, and the corneas engrafted with cellularized matrices eventually lose their transparency (Mimura et al., 2013). Introducing optimized biophysical cues into matrix design parameters may alleviate many challenges in recapitulating corneal function in these artificial constructs by improving functional protein expression. Endothelial cell morphology is dramatically improved on substrates imitating the stiffness of normal DM in comparison to softer or stiffer substrates (Palchesko et al., 2015). Similarly, cells grown on substrates presenting biomimetic topographic cues also exhibited differential protein expression. Immortalized human CEnCs grown on nanopillars, micropillars, and microwells displayed varied levels of the ZO-1 and Na^+/K^+-ATPase proteins, with the highest expression on substrates that minimized the cells' surface area (Koo et al., 2014).

The mechanical properties of carrier materials have been improved to produce substrates that are strong enough to survive cell culture and handling, but the provision of biophysical cues has not been adequately explored and this remains an understudied area. Incorporation of these features into the design of carrier substrates may be critical to developing grafts with long-term success.

7. Conclusion

Corneal endothelial cells, and the matrix that they secrete to produce the DM, have a bidirectional relationship. Corneal endothelial health relies on a normal and intact DM, and in turn healthy endothelial cells are necessary for the production of a normal extracellular matrix. This relationship in endothelial health and disease has been understudied and it critical that we consider the role of biophysical signals provided by the ECM. Fortunately, there are many tools available to characterize the cells' mechanical microenvironment and recapitulate it *in vitro* to provide a better model of what the corneal endothelium senses *in vivo*. The insights gleaned from investigating these interactions will expand our understanding of the pathogenesis of corneal endothelial dystrophies, and lead to improved outcomes for treatments.

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