Analytical Cellular Pathology

Applications and Challenges for Corneal Biology

Lead Guest Editor: Dimitrios Karamichos
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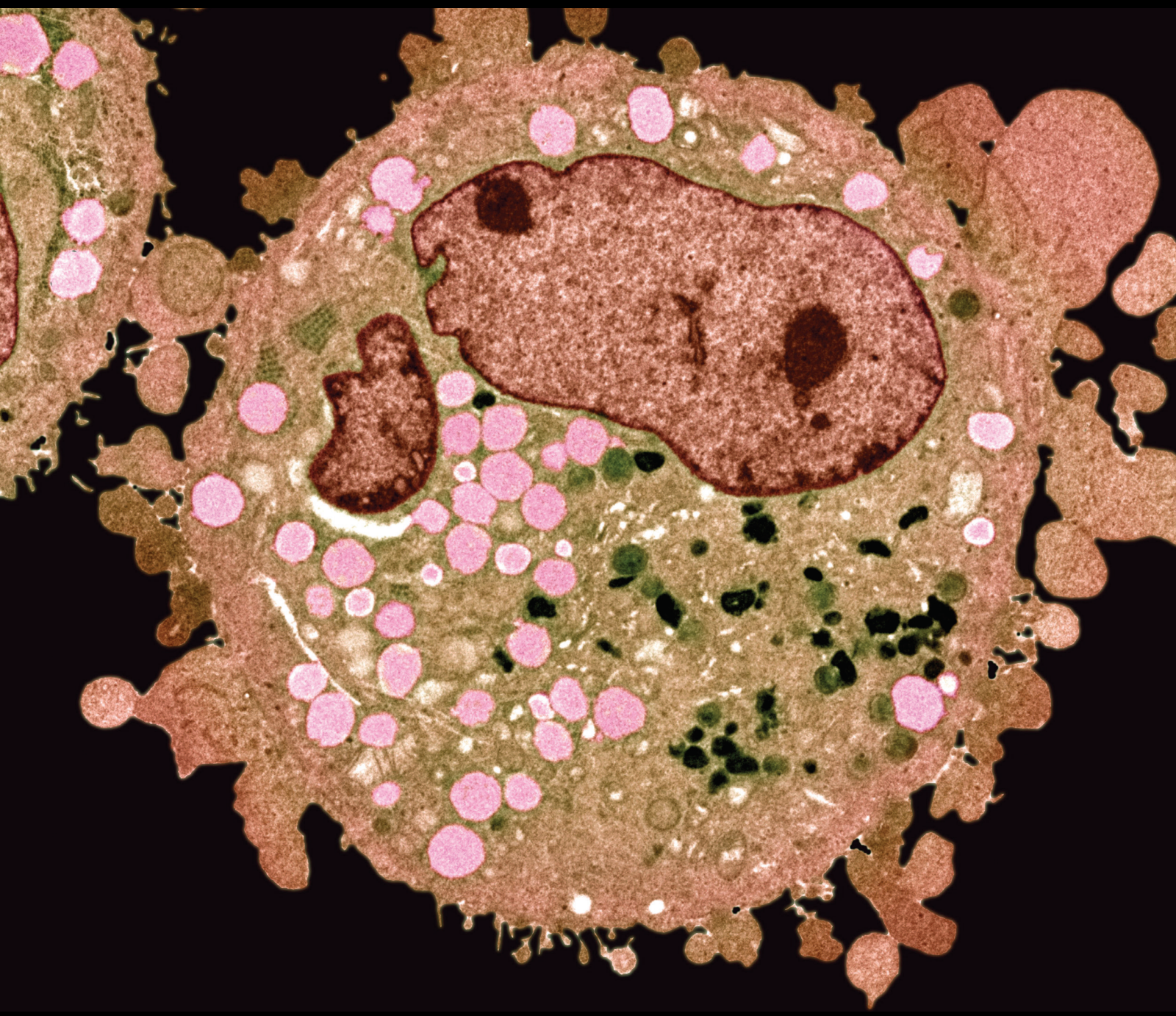

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Review Article

Extracellular Vesicles in the Cornea: Insights from Other Tissues

Tina B. McKay, Vincent Yeung, Audrey E. K. Hutcheon, Xiaoqing Guo, James D. Zieske,† and Joseph B. Ciolino

Department of Ophthalmology, Schepens Eye Research Institute of Mass Eye and Ear, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, USA
†Deceased

Correspondence should be addressed to Tina B. McKay; tmckay@mgh.harvard.edu

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Extracellular vesicles (EVs) are phospholipid bilayer-bound particles secreted by cells that have been found to be important in mediating cell-cell communication, signal transduction, and extracellular matrix remodeling. Their role in both physiological and pathological processes has been established in different tissues throughout the human body. The human cornea functions as a transparent and refractive barrier that protects the intraocular elements from the external environment. Injury, infection, or disease may cause the loss of corneal clarity by altering extracellular matrix organization within the stroma that may lead to detrimental effects on visual acuity. Over the years, numerous studies have identified many of the growth factors (e.g., transforming growth factor-β1, thrombospondin-1, and platelet-derived growth factor) important in corneal wound healing and scarring. However, the functional role of bound factors encapsulated in EVs in the context of corneal biology is less defined. In this review, we describe the discovery and characterization of EVs in the cornea. We focus on EV-matrix interactions, potential functions during corneal wound healing, and the bioactivity of mesenchymal stem cell-derived EVs. We also discuss the development of EVs as stable, drug-loaded therapeutics for ocular applications.

1. Introduction

Though their discovery dates back nearly three-quarters of a century, extracellular vesicles (EVs) have recently gained significant interest for their role in regulating physiological and pathological events important in human health and disease. An early study, published in 1946, first reported that specialized particles isolated from plasma following centrifugation possess diverse biological properties [1]. These sedimented particles were later described in 1967 as "platelet dust" that was clearly involved in blood coagulation [2]. It was in 1983 that Rose Johnstone's group applied immunogold labelling and transmission electron microscopy (TEM) to visualize the packaging of transferrin receptor into the multivesicular body and secretion of small EVs from a reticulocyte as it matured to a red blood cell [3, 4]. This foundational work, among others, paved the way in establishing EVs as distinct organelles that are secreted into the extracellular milieu in response to environmental changes and cell differentiation [5, 6]. This important finding, showing directed packaging of specific proteins into membrane-bound vesicles, sets the stage for later discoveries that defined fundamental roles for EVs in mediating cell-cell signaling, signal transduction, and extracellular matrix (ECM) remodeling in both physiological and pathological environments (reviewed by Yanez-Mo et al. [7]).

Since their discovery, EVs have since been classified into three broad subtypes based upon their biogenesis and size: exosomes (30-200 nm in diameter, packaged into the multivesicular body, and released by the endosomal pathway), microvesicles or ectosomes (100-1000 nm in diameter, arise from cell budding), and apoptotic bodies (0.5-2 μm in diameter, result of cell compartmentalization during cell death) [8]. The methods for categorizing different EV subclasses have traditionally focused heavily on size, predominantly exosomes and microvesicles. Though this dichotomization has been challenged, it remains the main classification system for EVs, as the complexity regarding definitive protein

markers and origin has not been clearly defined [9]. The International Society for Extracellular Vesicles endorses the term EVs, rather than exosomes or microvesicles, as the intracellular source and purity of the preparation are difficult to ascertain. General guidelines for using the term EVs are largely encouraged given the disparity in universal markers and the limitations in visualizing the formation of EVs in real time [10].

The molecular composition of EVs varies depending upon cellular origin and their biogenesis. EVs can incorporate a diverse repertoire of proteins, RNA, DNA, and lipids, which may lead to varied biological activity in the recipient cell. Characterization of EVs has been supported by the advancements of comprehensive databases (e.g., Vesiclepedia, ExoCarta, EVpedia, and exoRBase) that compile EV findings from numerous studies, with the aim of finding distinctive molecular signatures to specific cell/tissue types [11–13]. As a result, certain proteins, including classic exosomal markers, have been found to be present and may be used as EV markers: the tetraspanin proteins (CD9, CD63, and CD81), flotillin-1/-2, ESCRT-related genes (ALIX and TSG101), RABs, SNAREs, and others [14–16]. The larger microvesicles commonly share some protein markers that are found in exosomes, such as flotillin-1 and the major histocompatibility proteins (MHC-I and MHC-II), as well as enrichment of actinin-4 and mitofilin, among others [14]. Apoptotic markers, including Annexin V and C3b, have been identified on apoptotic bodies [17, 18]. EV markers independent of the cell of origin have also been reported, including ceramide enrichment, cholesterol, sphingomyelin, and other related lipids [19–21]. As the membrane of EVs is at least initially derived from the plasma membrane, EVs tend to retain the integrin markers of the cell of origin, which may contribute to delivering their bioactive cargo to specific cell types [22]. For corneal epithelial cells, these integrins include the $\beta1$ family of integrins ($\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, $\alpha9\beta1$, and $\alpha v\beta1$) [23, 24], with the $\beta1$ integrin subunit found in EVs secreted by these cells [25].

EVs have been detected in numerous biological fluids, including blood, lymph, saliva, urine, sweat, and tears [26–31], and are generally conserved throughout the animal kingdom from humans to microorganisms [32]. EVs are capable of mediating long-distance endocrine signaling, in addition to local paracrine or autocrine signals, depending on whether the EVs are endocytosed or secreted. For example, EVs released into circulation from adipose tissue have been shown to contain miRNAs that regulate gene and protein expression of metabolic factors within the liver and influence energy stores and glucose tolerance [33, 34]. Secretion of EVs during cardiovascular exercise likewise modulates liver function and may stimulate energy expenditure and metabolism [35]. In terms of pathology, EVs secreted from tumor cells have been found to be involved in the development, progression, and metastasis of certain cancers by actively transporting chemotherapeutics out of the cell [36] and priming a tumor-supportive microenvironment (reviewed by Shephard et al. and Webber et al. [37, 38]). The presence of oncogenes and onco-miRNAs within EVs may also confer resistance to select chemotherapeutics. Serum samples from HER2-positive breast cancer patients have been found to possess an abundance of EVs expressing HER2, a gene associated with promoting aggressive and metastatic cancer [39]. EV-mediated resistance to the antibody-based drug, trastuzumab, which selectively targets the overexpressed HER2 receptor has been reported [40]. These studies highlight the importance of EVs in short- and long-range cellular communication within the body and set the stage for a growing interest in EVs in human physiology.

2. Early Evidence of EVs in the Cornea

The cornea is composed of three major cellular layers—the corneal epithelium, stroma, and endothelium—and two acellular layers, Bowman's layer and Descemet's membrane, at the anterior and posterior regions, respectively. The bulk weight and volume of the cornea are made up by the hydrated stroma where resident keratocytes, immune cells, and nerve fiber bundles are dispersed in a highly organized ECM composed primarily by collagen types I and V. Blunt trauma, infection, chemical injury, and ocular or systemic disease may cause changes in ECM organization leading to corneal haze or scar development and visual impairment.

Dr. James D. Zieske, along with Dr. Ilene Gipson, published a notable paper in 1987 identifying the abundant expression of fibronectin following injury, which accounted for roughly 2% of the total protein within the wounded anterior stroma postkeratectomy [41]. In organ cultures, the source of the fibronectin was attributed to activated keratocytes since corneal epithelial cells appeared to express very little [41]. This data is consistent with studies showing increased fibronectin transcript levels by fibroblasts following injury [42]. This early study also described the appearance of membrane-bound particles within the anterior stroma by 3 days following a keratectomy, thus providing one of the first evidences for the presence of EVs in the cornea during wound healing (Figure 1). The use of the broad term "polysomes" in this landmark paper specified the diversity of membrane-bound microparticles observed in the wounded area in terms of cellular origin, cargo, and functionality that collectively may have very different effects depending on the subclass. More recently, we have found that human corneal epithelial cells express both fibronectin and thrombospondin-1 when cultured *in vitro*, with significant amounts also present in secreted EVs [25]. These EVs have been proposed to serve as a targeted source of fibronectin to the epithelial-stromal interface following wounding [43], though direct evidence of the release of bound provisional matrix proteins from EVs has not been shown to date.

In terms of marker expression, while the corneal epithelium shows high expression of the tight junctional protein, ZO-1, vesicles found within the epithelium do not appear to contain ZO-1, as assessed by immunogold TEM [44]. Similar to other cell types, EVs isolated from corneal epithelial cells have been found to contain the tetraspanin proteins, CD63 and CD9, as well as select laminin subunits (e.g., laminin α-3, α-4, β-1, γ-1, and γ-2) [25].

The presence of EVs secreted from the wounded epithelium has also been observed during basement membrane

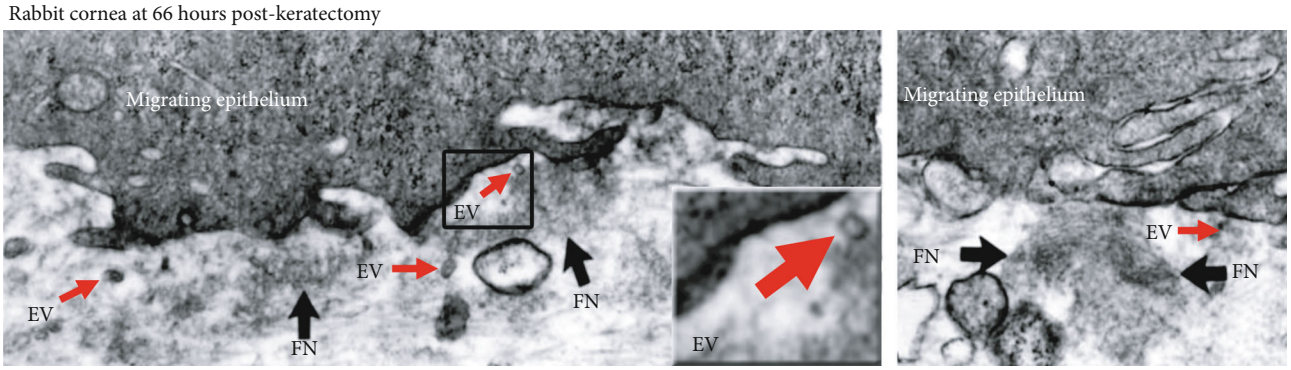

Figure 1: Transmission electron microscopy (TEM) images of a rabbit cornea 3 days postkeratectomy. The presence of EVs (*red arrows*) and fibronectin (FN, *black arrows*) is seen within the anterior stroma near the migrating epithelium. Original magnification: 31,200x. Inset magnification: 2.5x. Images were modified and reproduced from Zieske et al. 1987 [41] with permission from Dr. James D. Zieske (author), ARVO (copyright holder).

reformation. While a keratectomy removes the overlaying epithelium and basement membrane, along with a portion of the anterior stroma, a mild thermal burn to the ocular surface also leads to cell-mediated dissolution of the basement membrane via controlled release of matrix metalloproteinases [45, 46]. The migrating epithelium then deposits a fresh basement membrane, during which the formation of "blebs" become apparent at the basal edge of the epithelial cells [45]. These structures are the result of outward budding of portions of the plasma membrane that form microvesicles that may release bound cytoplasmic content into the extracellular milieu. Whether secretion of these EVs from the epithelium mediates reformation of the basement membrane is not clear; however, key basement membrane and provisional matrix proteins have been reported in EVs derived from corneal epithelial cells cultured *in vitro* [25].

3. EVs and Matrix Interactions

Current understanding regarding the biosynthesis of collagen in the cornea focuses on procollagen cleavage, lamellar organization, and ultimately, fibril cross-linking that leads to an ECM of sufficient stiffness to withstand external pressure, yet malleable and transparent to allow for vision (reviewed by McKay et al. and Meek and Knupp [47, 48]). The corneal stroma is composed of collagen fibrils with a relatively uniform diameter of ~25 nm. Collagen type V is known to serve a fundamental role in regulating collagen fibril diameter in the cornea by limiting the number of collagen type I monomers that may bind [49, 50]. Interactions between collagen and proteoglycans, such as lumican and decorin, also influence collagen fibrillogenesis and fibril diameter [51, 52]. These small collagen fibrils found in the cornea are thought to be required to permit complete tissue transparency and enable proper corneal curvature, elasticity, and rigidity.

Collagen fibrils, proteoglycans, and other ECM proteins play a concerted effort in regulating the binding and activation of secreted growth factors and, likewise, may influence EV migration and cell uptake. Studies in tissue-engineered corneal models have shown the presence of EVs distributed

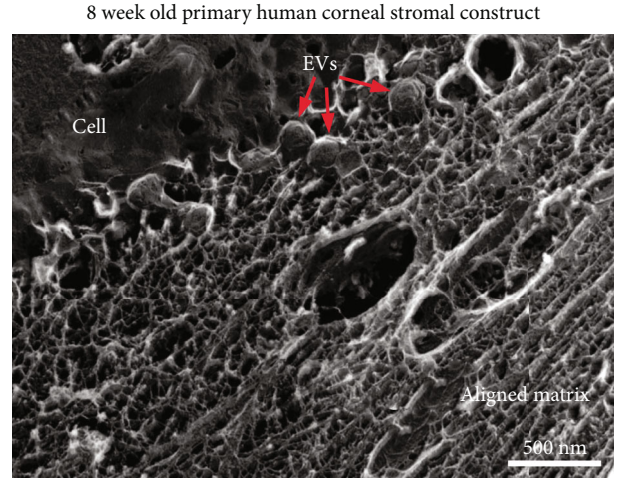

Figure 2: EVs (*red arrows*) budding from corneal fibroblasts (*cell*) in a self-assembled collagen matrix (*aligned matrix*). Image was modified and reproduced from Ren et al. 2008 [53] with permissions. Copyright (2008) Wiley-Liss, Inc.

within the collagen matrix [43]. The presence of EVs secreted by human corneal fibroblasts on the surface of a self-assembled ECM has been observed by TEM with maintenance of the characteristic rounded morphology (Figure 2).

Secretion and/or uptake of EVs have also been observed in a tissue-engineered model of the corneal stroma and corneal endothelium (Figure 3). This coculture model is constructed using human corneal fibroblasts that have secreted and assembled a collagen-rich matrix over a time period of one month followed by seeding of human corneal endothelial cells onto the mature stromal construct [54]. This model recapitulates corneal stromal-endothelial cell interactions found in the corneal tissue *in vivo* with a distribution of a self-assembled ECM and distal cell interactions [55]. The presence of EVs can be visualized within the matrix and localized between cell types supporting the application of these sophisticated tissue models in the study of EV-mediated cell-cell communication.

The use of quick-freeze/deep-etch (QFDE) electron microscopy has provided a unique method to evaluate

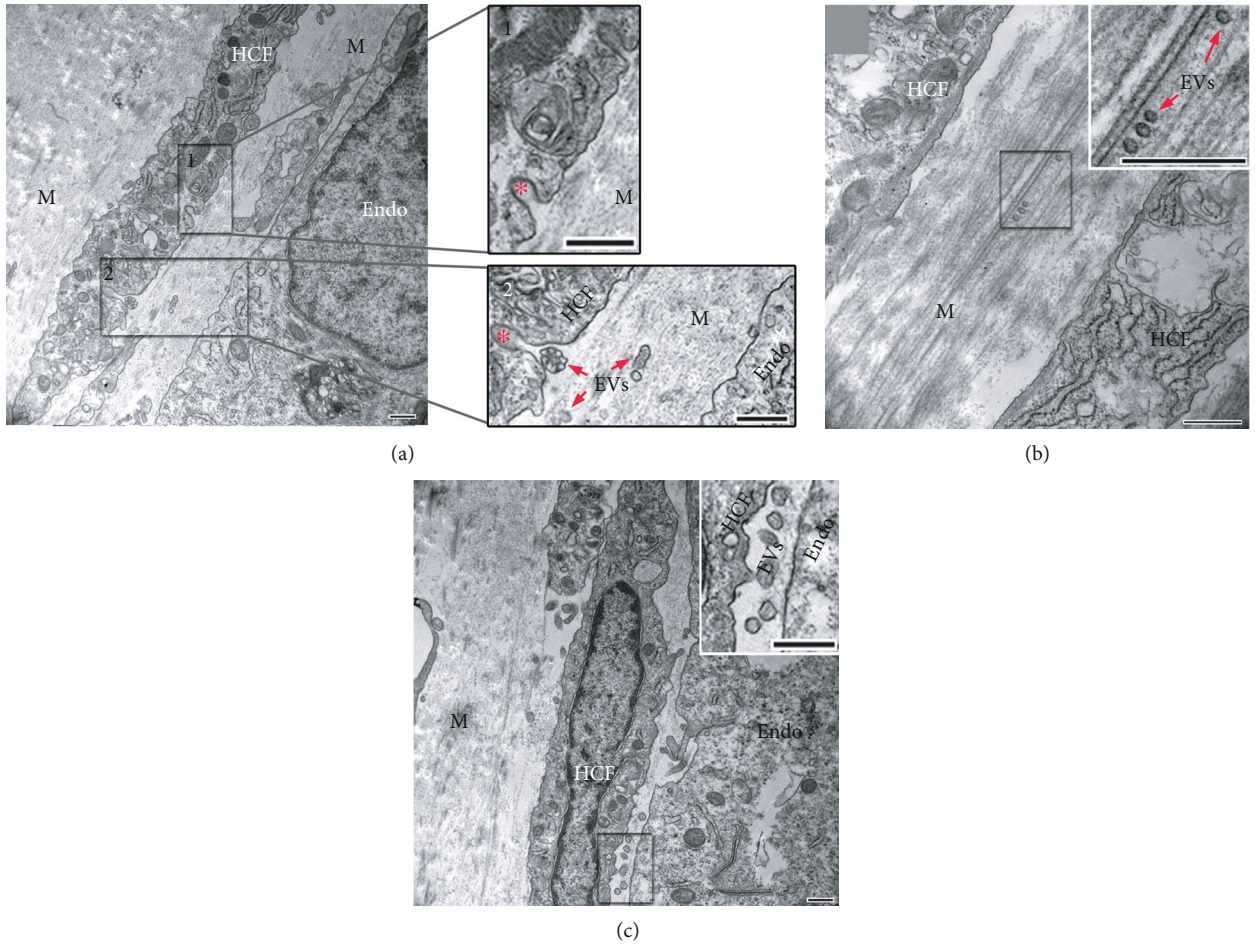

Figure 3: EVs distributed in a 3D tissue-engineered corneal endothelial-stromal model. (a) A coculture of human corneal fibroblast (HCF) and human corneal endothelial cells (Endo) shows (1) endocytosis/exocytosis (*red asterisks*) and (2) EVs (*red arrows*) present in the matrix (M) and a cluster of EV endocytosing/exocytosing. (b) EVs distributed within the stromal matrix. (c) EVs present between an HCF and Endo cell. Bars = 500 nm. Images were modified and reproduced from Zieske et al. (2020) [56] based on a Creative Commons BY license (CC BY 4.0), doi:10.1002/ar.24181.

collagen organization and EV interactions with collagen fibrils (Figure 4). An abundance of large and small EVs, presumably secreted by distant corneal fibroblasts, is distributed throughout the collagen matrix (Figures 4(a)–4(c)). The presence of polymerized fibrils emanating from a secreted EV provides supporting evidence that EVs may be a conduit for release of ECM components (Figure 4(d)). These findings are consistent with data from our lab showing an abundance of provisional matrix proteins, including fibronectin and thrombospondin-1, as well as basement membrane proteins, laminin and collagen type IV, present in corneal epithelial cell-derived EVs [25]. However, little is known regarding the mechanisms involved in the release of EV-bound proteins into the extracellular space.

Evidence in hard tissues, such as bone and cartilage, has provided clues to a potential role for EVs in mediating matrix deposition. Bone mineralization involves integration and deposition of minerals (e.g., calcium and phosphate ions) in the space between collagen type I fibrils, thereby forming a stiffened matrix to provide structural support for softer tissues. The presence of EVs rich in the catalytic enzyme, alkaline phosphatase, has been observed in bone and regions of cartilage growth [58–61]. Alkaline phosphatase is expressed in a number of tissues in the human body, including the bone, liver, and intestine, and functions as an enzyme for phosphate monoester substrates to generate an alcohol and monophosphate ion. One of the major components in hydroxyapatite crystals is inorganic phosphate, which forms a complex with calcium. The following different mechanisms have been proposed for the functional role of these EVs in mediating calcification: regulation of phosphate levels, promotion of apatite crystal formation, and binding interactions with the collagen and proteoglycans in bone or cartilage [62]. These EVs are highly electron dense and form hydroxyapatite that may serve as a nucleation site for matrix deposition [63]. However, whether this phenomenon occurs during ECM formation and/or remodeling in soft tissues, such as the cornea, remains unclear.

4. EVs and Wound Healing

EVs have been found to be secreted in the cornea following wounding [41, 64, 65]. In an epithelial debridement model, numerous EVs were shown to be present at the basal side

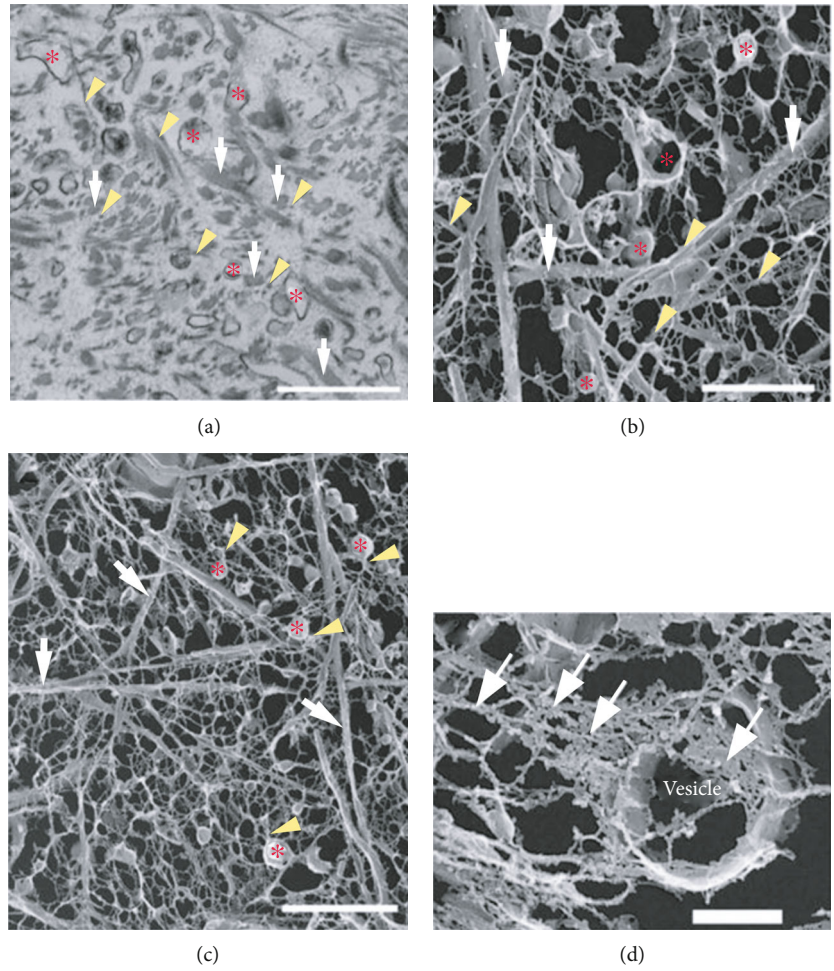

Figure 4: EV-collagen interactions in a reconstituted collagen substrate. (a) TEM image of EVs (*red asterisks*) distributed within the collagen matrix. A number of large collagen aggregates are attributed to PureCol collagen (*white arrows*) with the presence of small fibrils (*yellow arrowheads*). Scale bar = 1 μm. (b) High-magnification QFDE image shows EVs (*red asterisks*), large fibril bundles (*white arrows*), and small fibril bundles (*yellow arrowheads*) present within the collagen matrix. Scale bar = 1 μm. (c) Low-magnification QFDE image shows EVs (*red asterisks*) connected with small fibril bundles (*yellow arrowheads*). Large fibril bundles (*white arrows*) were also present. Scale bar = 2 μm. (d) High-magnification QFDE image of an EV (*vesicle*) containing matrix components (*white arrows*). Scale bar = 0.5 μm. Images were modified and reproduced from Saeidi et al. 2012 [57] based on a Creative Commons license, doi:10.1002/bit.24533.

of the migrating epithelium and apical to the basement membrane (Figure 5(a)). Interestingly, the epithelial basement membrane appeared to limit the diffusion of these epithelial EVs to the stroma [56, 64] in a manner similar to that observed with growth factors found in the tear film. These growth factors, such as transforming growth factor-beta1 (TGF-β1), typically correlate with corneal scarring following injury where the basement membrane is damaged [65, 66]. As seen in Figure 5(b), when the basement membrane is removed by keratectomy, EVs appear to pass into the stroma and potentially communicate with the stromal cells or matrix. For TGF-β1 and related growth factors, binding of the TGF-β prodomain may occur with basement proteins, such as perlecan and nidogen-1, mediated via electrostatic interactions and chemical association heparin sulfate proteoglycans [67]. These interactions have been observed in *in vitro* systems using binding assays to assess protein affinity in a static or microfluidic environment. Restricted permeability of the corneal epithelial basement membrane to TGF-β1 has been purported as a protective measure to prevent corneal scarring [66, 68]. A similar binding interaction involving epithelial cell-derived EVs and the epithelial basement membrane may also explain the resistance to scarring in debridement models when the basement membrane remains intact compared to following a keratectomy, in which the basement membrane is removed, and scar development is more common.

Similar to the fibrillar material contained within EVs shown in a tissue-engineered stromal system [57], secreted EVs may also contain basement membrane proteins. We have found that isolated EVs secreted by a human corneal epithelial cell line promote myofibroblast differentiation when applied to corneal fibroblasts cultured in a 3D *in vitro* stromal model [25]. Protein analysis of isolated corneal epithelial cell-derived EVs has identified proteins associated with provisional matrix, such as thrombospondin-1 and fibronectin, suggesting that EVs may contain proteins associated with basement membrane reformation [25]. We have

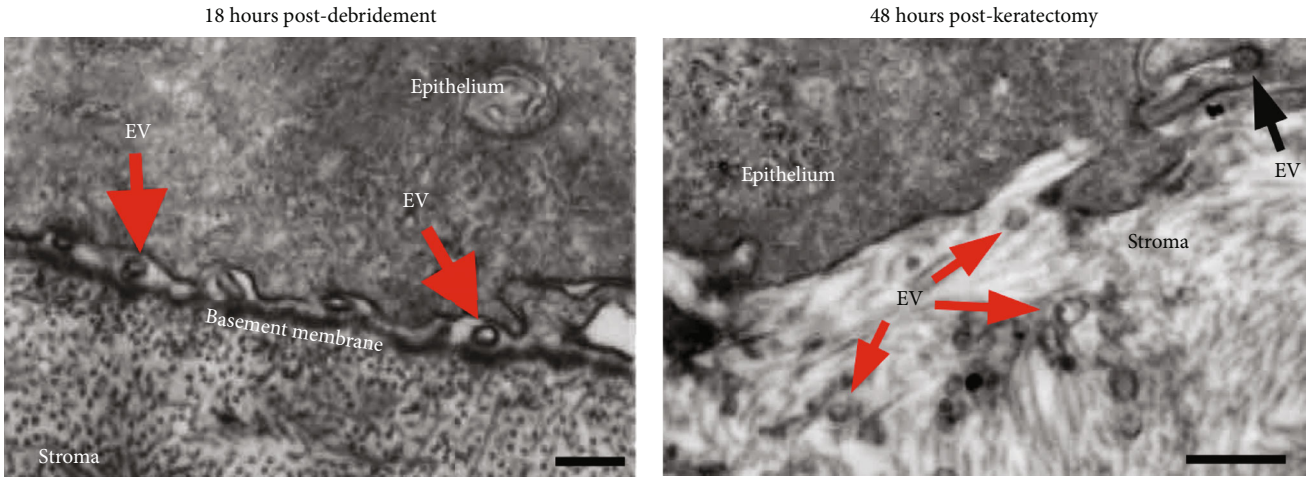

Figure 5: TEM images of the epithelial-stromal interface in the corneas with varying severity of wounds: (a) debridement (basement membrane left intact) and (b) keratectomy (basement membrane removed). (a) Localization of EVs (*red arrows*) on the anterior side of the epithelial basement membrane in a rat cornea at 18 hours postdebridement. Scale bar = 250 nm. (b) Dispersion of EVs (*red arrows*) in the anterior stroma in a rabbit cornea at 48 hours postkeratectomy. EV undergoing endocytosis/exocytosis (*black arrow*). Scale bar = 250 nm. Images were modified and reproduced from Han et al. 2017 [64] based on a Creative Commons license (CC BY 4.0), doi:10.1038/srep40548.

found that EVs isolated from human corneal endothelial cells also contain basement membrane proteins, including laminin and heparan sulfate proteoglycan core proteins (*unpublished data*), suggesting that EVs may also be conduits for transfer and assembly of the basement membrane from neighbouring cells. It could be hypothesized that EV-associated heparan sulfate proteoglycans are responsible for EV internalization into their targeted recipient cells and contribute to their functional activity [69]. These studies provide evidence that the deposition of a proper corneal epithelial basement membrane, at least *in vitro*, requires the presence of stromal and/or corneal endothelial cells [70]. Biochemical analyses have shown that both corneal keratocytes and fibroblasts express epithelial basement membrane proteins, such as perlecan and nidogen-2, *in vitro* [71], suggesting that corneal stromal fibroblasts cells may serve as a key source of basement membrane proteins following epithelial injury to accelerate basement membrane reformation. Notable questions remain, however, regarding the role of EVs as stable carriers of basement membrane proteins and the mechanism(s) by which these rather large proteins escape the bilayer membrane of EVs.

5. Mesenchymal Stem Cell-Derived EVs

Numerous studies have described the antifibrotic properties of mesenchymal stem cell- (MSC-) derived EVs in the cornea and skin [72]. The antiscarring properties of MSCs have been at least partially attributed to microRNAs encapsulated within EVs. Of particular interest, the immunomodulatory properties of MSC-derived EVs suggest that these vesicles may be useful in improving patient-centered outcomes following corneal transplantation.

Our current understanding of MSCs isolated from bone marrow [73] and other adult tissues (e.g., adipose, Wharton's jelly, and cornea) [74–76] is that they have the capacity to differentiate into mesoderm-derived lineages that possess regenerative, reparative, and immunomodulatory properties. By meeting the minimum framework of human MSCs as defined by the International Society for Cellular Therapy (ISCT) [77], the therapeutic application of MSCs has been highlighted to be effective on a wide range of animal models, to reduce corneal scarring [78, 79], restore corneal transparency [80], and exert corneal antifibrotic effects [81, 82].

Indeed, the early reports of MSC multipotent differentiation capacity fuelled the initial enthusiasm for the new regenerative paradigm by donor cell engraftment. Subsequent studies, however, clarified that paracrine factors play a huge role, in the mechanism of MSC therapeutic action, verified in many independent studies targeting a variety of different tissues, including the kidney, heart, nervous tissues, skeletal muscle, eyes, lung, and placenta [83–91].

With the focus of administering MSC-derived EVs on different corneal disease models, it was reported that topical application of corneal MSC-derived EVs accelerated corneal epithelial wound healing [92], decreased corneal epithelial defects, and reduced inflammatory cytokine production in mice with desiccating stress [93]. Significantly, and congruently, the enhanced proliferation and suppressed apoptosis, as well as the suppressed proinflammatory properties in corneal epithelial cells treated with MSC-derived EVs, confer a similar reparative effect on corneal wound repair [94] as induced pluripotent stem cell-derived EVs [95]. In addition, MSC-derived EVs were observed to alter corneal stromal cells by promoting ECM synthesis, changing matrix metalloproteinases and collagen levels, and increasing stromal cell

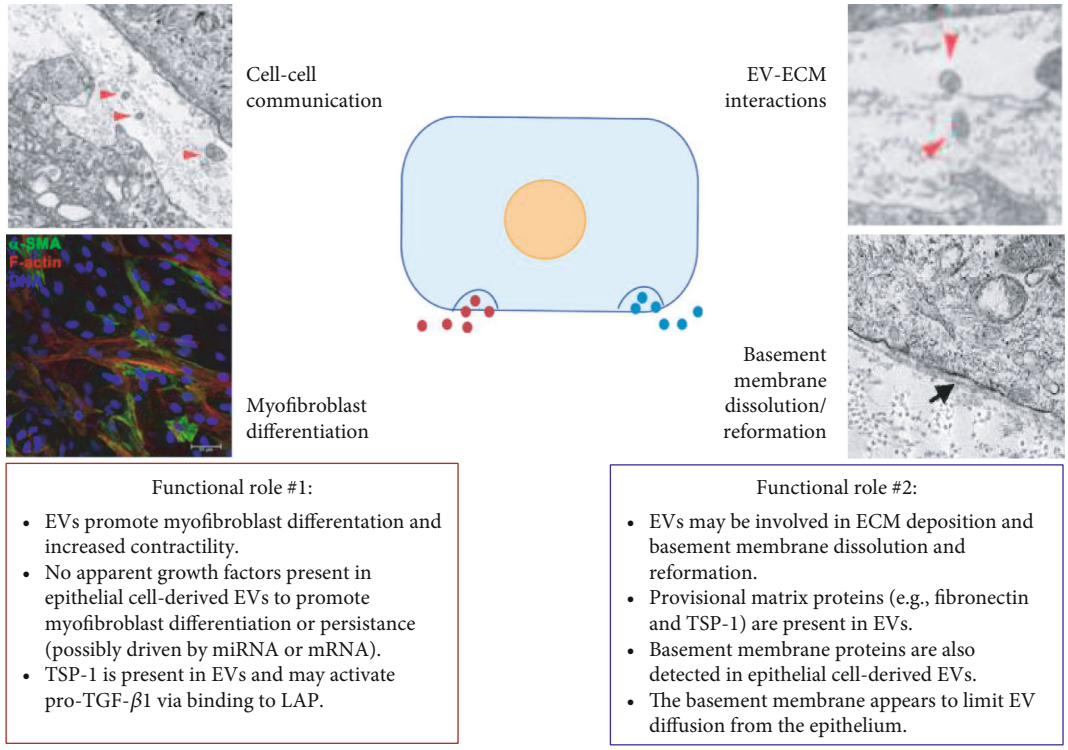

Figure 6: Proposed functional roles of EVs in the cornea. The presence of EVs (*red arrowheads*) dispersed in the ECM of a self-assembled corneal fibroblast construct. A basement membrane (*black arrow*) forms between corneal epithelial cells and the stromal matrix. Images were modified and reproduced from McKay et al. (2019 and 2020) [25, 43] based on a Creative Commons BY license (CC BY 4.0), doi:10.3390/cells9051080 and doi:10.3390/bioengineering6040110.

proliferation [96]. A collective effort remains in deciphering the therapeutic mechanism of MSC-derived EVs. One study reported that corneal stem cell-derived EVs lacking unique miRNA sets (miR-23-3p, miR-191-3p, miR-221-39, and miR-222-3p) were ineffective in reducing inflammation and blocking corneal scarring after wounding [97]. This strongly suggests that MSC-derived EV treatment for corneal-related injuries may prove to be efficacious in restoring homeostasis at the onset of injury.

6. Drug-Loaded EVs as Therapeutics

Within the past decade, EVs have emerged as an attractive candidate for the new generation of a natural nanoscale delivery system. Attributed to their intrinsic ability to internalize an array of antigens to elicit a biological response to target cells, a multitude of studies have focused on exploiting EVs as therapeutic carriers. These EVs are ideal nanoscale carriers for potential clinical applications owing to their capacity to avoid rapid clearance by the mononuclear phagocyte system [98], overcoming immunotoxicity [99], and staying in the body's circulation longer due to the negative zeta potential or deeper penetration into tissues due to their size [100].

The main approaches for loading therapeutic cargo (e.g., functional RNA, DNA molecules, peptides, and synthetic drugs) include active and passive encapsulation. The active cargo approach involves the temporary disruption of the EV plasma membrane by sonication or electroporation [101]. In contrast, the passive method uses diffusion, where drugs (e.g., paclitaxel and curcumin) load along the concentration gradient, depending on their hydrophobicity [102]. Many studies predominantly focused on loading therapeutics into EVs in the field of oncology. This was initially reported with loading curcumin into EVs derived from various cell types (e.g., mouse embryonic fibroblasts, mouse lymphoma, and human adenocarcinoma cells), which showed better solubility and anti-inflammatory bioactivity compared to traditional curcumin administration [103, 104]. This study paralleled those demonstrating that loading paclitaxel into MSCs [105], LNCaP or PC-3 prostate cells [106], or taxol into MSCs [107] resulted in enhanced cytotoxicity and inhibition of tumor cell growth. There have been numerous studies [101] showcasing chemotherapeutic drug loading into EVs, showing higher efficacy and superior bioavailability, but research has been sparse regarding ocular pharmacology.

One study reported that EV-associated adenoassociated virus type 2 (EV-AAV-2) from 293 T cells demonstrated deeper penetration via intravitreal injection in the retina, efficiently reaching the inner and outer plexiform compared to conventional AAV-2, thereby suggesting that this treatment may be an effective method for intravitreal gene transfer into the retina [108]. Furthermore, another study showed that MSC-EVs overexpressing miR-126 successfully suppressed the HMGB1 signaling pathway and suppressed hyperglycemia-induced retinal inflammation in rats [109]. So far, however, there has been limited research on loading therapeutic cargo onto EVs to treat eye-related diseases or

injury. Therefore, further endeavours are required to develop novel therapies in ophthalmology.

7. Conclusions

The laboratory of Dr. James D. Zieske, and many others, has made pivotal discoveries over the years investigating the fundamental mechanisms involved in corneal wound healing. This work includes the initial discovery of EVs in the wounded cornea in 1987, with the observation of EV-collagen interactions nearly twenty years later, and finally, the proteomic and functional characterizations of epithelial cell-derived EVs in 2020. The development and application of biologically relevant tissue-engineered models of the human cornea, including 3D self-assembled stromal models and epithelial- and endothelial-stromal cocultures, provided further evidence regarding the key players that may promote corneal scarring (e.g., TGF-β1, platelet-derived growth factor, and epithelial cell-derived EVs) and those that may inhibit scarring (e.g., TGF-β3 and mitomycin C). The growing interest in EVs by the Zieske group through the years may perhaps be linked to their innovative and early use of high-resolution imaging (TEM and confocal microscopy) to visualize changes in corneal tissue structure and protein expression in different animal and tissue models, a constant theme from this group that helped define the temporal cascade involved in corneal wound healing. The discovery of the presence of these submicron-, phospholipid bilayer membrane-bound particles in the cornea occurred with the visualization of corneal tissue sections by electron microscopy, in a similar manner to how they were originally discovered in reticulocytes around the same time. EVs secreted from the corneal epithelium in response to wounding were eventually identified and appear to be localized to the anterior basement membrane following debridement, but not following a keratectomy. Largely absent in the uninjured cornea, the abundance of EVs in the anterior cornea following superficial wounding prompted the supposition that EVs secreted by the corneal epithelium in response to tissue damage may have an important function during corneal repair.

Consistent with the diverse properties of EVs in other tissues throughout the body, EVs derived from—and secreted within—the cornea also appear to mediate various functional outcomes that may influence cell phenotype (e.g., myofibroblast differentiation), ECM structure (e.g., matrix contraction and fibrotic composition), and epithelial-stromal interactions (e.g., basement membrane dissolution and reformation) (Figure 6). It is likely that different subpopulations of corneal EVs have diverse effects depending on the cell of origin, relative abundance, and the target cell. Parsing out these different functional roles of EVs in the cornea will require careful isolation and biochemical analyses to define reproducible surface markers consistent with specific EV subpopulations and their associated properties (i.e., EVs that promote myofibroblast differentiation compared to EVs that carry provisional matrix proteins). Likewise, identifying how wounding or disease influence EV composition and secretion may help in understanding corneal tissue regeneration and fibrosis and the underlying mechanisms that determine the clinical outcome (e.g., scarless healing or scar development). The antifibrotic and immunomodulatory properties of MSC-derived EVs provide solid evidence that isolated EVs may serve as a targeted therapeutic approach to promote corneal wound healing. Clearly, further studies are needed to provide mechanistic insight into the role of EVs in the cornea in the context of wound healing and disease. This work is technically challenging, but no doubt will help advance our understanding of cell communication in the cornea and the role of the microenvironment in tissue regeneration.

Data Availability

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

In loving memory of Dr. James D. Zieske, without whom this review would never have been conceptualized. His ongoing curiosity of cell-cell communication led him to explore the idea that EVs are highly involved in the physiological and pathological scenarios encompassed within the corneal stromal milieu. Jim was not only a leader in the field of corneal biology, but he was also a beloved friend, mentor, peer, and employer. Jim, you made a huge impact on the scientific community, as well as our lives. We will endeavour to continue your scientific research in both practice and spirit. You are sorely missed! This research was funded by the National Eye Institute, National Institutes of Health (5T32EY007145-20, R01EY005665, and the core grant P30EY003790).

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Research Article

Pannexin1: Role as a Sensor to Injury Is Attenuated in Pretype 2 Corneal Diabetic Epithelium

Garrett Rhodes,[1] Kristen L. Segars,[2] Yoonjoo K. Lee,[2] Audrey E. K. Hutcheon,[3] Celeste B. Rich,[4] and Vickery Trinkaus-Randall [1,4]

[1]*Department of Ophthalmology, Boston University School of Medicine, Boston, Massachusetts 02118, USA*
[2]*Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts 02118, USA*
[3]*Schepens Eye Research Institute of Mass Eye and Ear, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, USA*
[4]*Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118, USA*

Correspondence should be addressed to Vickery Trinkaus-Randall; vickery@bu.edu

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Epithelial wound healing is essential to repair the corneal barrier function after injury and requires coordinated epithelial sheet movement over the wounded region. The presence and role of pannexin1 on multilayered epithelial sheet migration was examined in unwounded and wounded corneal epithelium from C57BL/6J (B6) control and diet-induced obese (DiO) mice, a pretype 2 diabetic model. We hypothesize that pannexin1 is dysregulated, and the interaction of two ion-channel proteins (P2X7 and pannexin1) is altered in pretype 2 diabetic tissue. Pannexin1 was found to be present along cell borders in unwounded tissue, and no significant difference was observed between DiO and B6 control. However, an epithelial debridement induced a striking difference in pannexin1 localization. The B6 control epithelium displayed intense staining near the leading edge, which is the region where calcium mobilization was detected, whereas the staining in the DiO corneal epithelium was diffuse and lacked distinct gradation in intensity back from the leading edge. Cells distal to the wound in the DiO tissue were irregular in shape, and the morphology was similar to that of epithelium inhibited with 10Panx, a pannexin1 inhibitor. Pannexin1 inhibition reduced mobilization of calcium between cells near the leading edge, and MATLAB scripts revealed a reduction in cell-cell communication that was also detected in cultured cells. Proximity ligation was performed to determine if P2X7 and pannexin1 interaction was a necessary component of motility and communication. While there was no significant difference in the interaction in unwounded DiO and B6 control corneal epithelium, there was significantly less interaction in the wounded DiO corneas both near the wound and back from the edge. The results demonstrate that pannexin1 contributes to the healing response, and P2X7 and pannexin1 coordination may be a required component of cell-cell communication and an underlying reason for the lack of pathologic tissue migration.

1. Introduction

Obesity is a major risk factor for the development of type 2 diabetes. The cornea is an excellent organ to examine the effects of diet on wound healing, as it is a relatively simple avascular tissue that is oxygenated via diffusion. Corneal epithelial repair requires a number of synchronized events that include, but are not limited to, cell motility, cell-cell communication, matrix deposition, and tissue remodeling [1, 2]. In diabetes, the apical corneal epithelium and its tight junctions become compromised, and large molecules and pathogens can penetrate into the epithelium. This impaired function appears to be exacerbated in patients with elevated HbA1c levels [3–5] and enhances the risk for delayed wound healing after injury, epithelial fragility, and possibly decreased corneal epithelial stiffness [6–8].

One of the earliest demonstrations of injury response was performed by live-cell imaging of rabbit corneal epithelial cells. In this study, it was observed that the injury-induced release of nucleotides triggered a transient calcium (Ca^{2+})

mobilization near the wound site that crossed acellular zones, indicating that it was independent of gap junctions [9]. In addition to the initial release of ATP, factors such as EGF, cytokines, and other nucleotides were released from tears, activated growth factor receptors, and other proteins [10, 11]. This ATP binds and activates a large family of transmembrane purinoreceptors (P2Y and P2X receptors) in the cornea resulting in downstream signaling [9, 11, 12]. The G-protein P2Y receptors cause an increase in intracellular Ca^{2+}, while the P2X receptors are ion channels and gate calcium and other ions [13, 14]. One P2X receptor, P2X7, is often considered to be a pain receptor, is expressed by both corneal epithelium and nerves [15, 16], and mediates cell migration [17]. Under normal physiological conditions, P2X7 localization is intense in the epithelial cells near the leading edge of the wound; however, once the edges of the wound have made contact and the epithelium restratifies, its localization realigns along the cell borders [17]. In vitro studies revealed that activation of the P2X7 receptor elicits a unique Ca^{2+} mobilization profile that travels throughout the epithelial sheet in a predictive cyclic pattern displaying cell-cell communication not through gap junctions but through pannexin channels [18].

Studies on diabetic corneal epithelium revealed a significant increase (4-7-fold) in P2X7 mRNA compared to age matched individuals [15, 17]. In addition, there is a report that patients with type 2 diabetes had increased P2X7 in peripheral blood mononuclear cells compared to control individuals [19]. Previous studies on corneal epithelium from a pretype 2 diabetic obese murine model demonstrated that there was a loss of sensory epithelial nerves and a change in P2X7 localization in the epithelium and stroma after corneal abrasions [6]. Furthermore, investigations have shown that interaction of P2X7 and pannexin1, another ion channel, was present in cultured corneal epithelial studies, while other studies showed that P2X7 knockout mice displayed reduced pannexin1 [18, 20, 21]. These data indicate that both pannexin1 and P2X7 may act as sensing proteins near the leading edge of the wound.

The pannexins belong to a family of large pore proteins that transport and release intracellular ATP into the interstitial space through a single membrane channel, and in so doing, play a number of roles in signaling, inflammation, and cytoskeletal reorganization [22, 23]. Researchers have hypothesized that ATP moves through pannexin channels and activates P2X receptors in a feed-forward pattern [24]. Recently, we detected pannexin1 near the leading edge of the wound in cultured epithelial cells and demonstrated that P2X7 receptor activation was abrogated by pannexin1 inhibition [18]. Taken together, these results suggest that epithelial cells display a complex repertoire of communication between pannexin1 and P2X7.

The major goals of this study were to determine if there was a difference in the localization of pannexin1 and the association between P2X7 and pannexin1 in C57BL/6J (B6) control and pretype 2 diabetic (DiO) murine corneal epithelium. We hypothesize that pannexin1 facilitates the healing process through its localization near the leading edge, and the subsequent movement of ATP through its channels activates P2X7 receptors, thus promoting migration. While pannexin1 localization was similar in unwounded corneal epithelium from both tissues, there were striking differences after injury. After 2 hours, pannexin1 in the region adjacent to the leading edge was intense in B6 control and diffuse in DiO; however, after 20 hours, this response was less intense in both the B6 control and DiO tissue. When pannexin1 was inhibited, there was a 50% decrease in cell-cell communication, compared to B6 control. Furthermore, proximity ligation assays (PLA) demonstrated that while P2X7 and pannexin1 displayed a similar level of interaction in unwounded corneas, there was a significant decrease in association in the wounded DiO corneal epithelium. The potential synergistic relationship and physical association indicate that pannexin1 may be a potential target for pharmacological intervention of wounds.

2. Materials and Methods

2.1. Antibodies and Inhibitors. For immunohistochemistry, anti-pannexin1 polyclonal rabbit antibody directed against pannexin1 (Cat. #ACC-234) and anti-P2X7 polyclonal rabbit antibody targeting the P2X7 receptor (Cat. #APR-004) were purchased from Alomone Labs (Jerusalem, Israel). For proximity ligation assay, anti-pannexin1 monoclonal mouse antibody directed against pannexin1 (Cat. #SC-515941) was purchased from Santa Cruz Biotechnology (Dallas, TX), and anti-P2X7 polyclonal rabbit antibody targeting the extracellular domain of P2X7 receptors (Cat. #APR-008) was purchased from Alomone Labs. 10Panx, a pannexin1 mimetic inhibitory peptide (Cat. #3348), and its scrambled peptide were purchased from Tocris Biosciences (Minneapolis, MN). Alexa Fluor-conjugated Plus 488 donkey anti-rabbit and mouse IgG secondary antibodies and rhodamine-phalloidin were purchased from Invitrogen (Carlsbad, CA).

2.2. Tissue Preparation and Organ Culture. All mice were purchased from Jackson Laboratory (The Jackson Laboratory; Bar Harbor, ME), and research protocols conformed to the standards of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic Care and Vision Research, as well as the Boston University IACUC. The C57BL/6J mice were fed either a control diet (B6 control) or a high fat diet to induce obesity (DiO) (Jackson Laboratory): Control Diet D12450B (10 kcal% fat, 3.8 kcal/g) and High Fat Diet D12492 (60 kcal% fat, 5.2 kcal/g) [6].

To examine localization of P2X7 and pannexin1 in response to injury, 1.5 mm debridement wounds were performed on both B6 control and DiO mice, as previously described [6, 17, 18]. After debridement, the mice were euthanized, the eyes enucleated, and placed in organ culture [12, 17], and incubated in serum-free Keratinocyte medium (Invitrogen Carlsbad, CA) containing 100 μg/mL penicillin and 100 μg/mL streptomycin for 2, 8, or 20 hours at 37°C and 5% CO_2. A minimum of 3 eyes per time point per condition was analyzed.

2.3. Immunofluorescence. After each time point, the globes were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 30 minutes at room temperature (RT). Immunofluorescent staining was performed [17] by permeabilizing and blocking the tissue in blocking solution (PBS (93% *v*/*v*), Triton X-100 (10% *v*/*v*), BSA (5% *v*/*v*)) for one hour (hr) at RT. The tissue was incubated in primary antibody diluted in blocking solution overnight at 4°C (dilution will be described with each experiment) and washed with PBS. Alexa Fluor-conjugated 488 secondary antibodies (Invitrogen, Carlsbad, CA) were used at a dilution of 1 : 300 in blocking solution for 1 hr at RT prior to being washed again with PBS. The tissue was counter-stained with rhodamine-conjugated phalloidin (Invitrogen, 1 : 50) to visualize F-actin. Cells or tissues were mounted using Vecta-SHIELD containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA) to visualize cell nuclei. The primary antibody was excluded from the secondary control tissue. Following staining, the corneas were imaged using confocal microscopy.

2.4. Confocal Microscopy for Immunofluorescence. The tissue was prepared by slicing the cornea into radial sections and cutting butterfly slits into the scleral rim in order to flatten the tissue for a uniform image [17]. Tissues were sandwiched between a p35 MatTek tissue culture dish (MatTek; Ashland, MA) and a glass coverslip, and imaged enface from the apical epithelium toward the stroma on a Zeiss LSM 700 (Zeiss, Thornwood, NY) confocal microscope. Fluorescent gain levels were set using secondary control samples, with the pinhole size maintained at 1 Airy unit. The settings and objectives were not changed across all images in order to eliminate changes in data due to varying confocal settings. Each region was collected as a *Z*-stack in 1 μm steps and included the apical and basal cells.

2.5. Proximity Ligation Assay (PLA). Detection of proteins by proximity ligation assay (PLA) was performed using an anti-pannexin1 monoclonal mouse antibody and an anti-P2X7 polyclonal rabbit antibody that targets the extracellular domain of the P2X7 receptor. Detection was carried out using Duolink® reagents (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's Duolink® PLA Fluorescence Protocol with slight modifications. The donkey anti-mouse MINUS (DUO92004) and donkey anti-rabbit PLUS (DUO92002) PLA probes, along with Duolink® blocking solution, antibody diluent, ligation buffer, amplification buffer, and specific wash buffers, were used in the assay. Tissue sections were incubated in blocking buffer for 60 minutes at 37°C in a preheated humidity chamber. Antibodies were diluted 1 : 100 (anti-pannexin1) and 1 : 300 (anti-P2X7) in antibody diluent, applied to tissue, incubated for 2 hrs at RT, and then washed in 1x Wash Buffer A at RT. PLUS and MINUS probes were diluted in antibody diluent mixture, and sections were incubated for 1 hr at 37°C in the heated humidity chamber. Sections were washed in Wash Buffer A at RT. The ligation buffer was mixed in high purity water, and ligase was added and incubated for 30 minutes at 37°C in a heated humidity chamber. Sections were washed in Wash Buffer A at RT. Sections were incubated in amplification buffer containing polymerase for 100 minutes at 37°C in a heated humidity chamber. Sections were washed in Wash Buffer B at RT and then with 0.01x Wash Buffer B. The tissue was imaged on the Zeiss Axiovert LSM 700.

2.6. Analysis of Proximity Ligation. Images were taken both near the leading edge and contiguous regions back from the edge using both the tiling function and optical slices. Analysis was conducted with ImageJ and CellProfiler (Broad Institute, Cambridge MA) to determine if DNA ligation occurred by assessing fluorescence, which only occurred if the PLUS and MINUS PLA probes were within 40 nm of each other [25]. To analyze for puncta, the images were imported into ImageJ; an initial thresholding was performed to separate puncta from background noise, and the file was exported into CellProfiler. The Spot Detection pipeline was used to count puncta. The size of an average puncta was determined using the measure tool in ImageJ and refined in CellProfiler. For consistency, the same analytical parameters were used for all images. Statistical analysis was performed using GraphPad Prism 5.

2.7. Calcium (Ca^{2+}) Mobilization. Ca^{2+} mobilization experiments were performed on ex vivo mouse corneas [18]. In brief, the corneas were preincubated in Cal-520 AM and CellMask™ Deep Red (Thermo Fisher, Waltham, MA) for 30 minutes at 37°C and 5% CO_2. Cal-520 AM was used at 1 : 100, and CellMask Deep Red was used at 1 : 10,000, and the final concentration of DMSO and pluronic acid was 1% (*v*/*v*) and 20% (*w*/*v*), respectively. Excess dye was removed, and the corneas were mounted in polyethylene glycol on silanized glass bottom dishes [26]. For in vitro imaging, human corneal epithelial (HCE) cells [17] were cultured to confluence on glass bottom dishes and preloaded with the calcium indicator dye. Images were collected every 5 seconds for 45 minutes on a Zeiss Axiovert LSM 880 confocal microscope (Carl Zeiss, White Plains, NY) utilizing the FAST module and AIRYScan.

2.8. Modeling of Ca^{2+} Waves. Image analysis was performed using FIJI/ImageJ (NIH, Bethesda, MD) and MATLAB scripts (MATLAB, MathWorks, Inc.) that were written specifically for the analysis of Ca^{2+} waves, as previously described [12, 18, 27]. In brief, videos from each experiment were exported as TIF or AVI format, imported into the custom MATLAB scripts, and analyzed for Ca^{2+} responses based on cell population (individual cells or groups of cells). These specific MATLAB scripts enabled the evaluation of the spatiotemporal and cell-cell communication of these cell populations by identifying Ca^{2+} events, generating an event kymograph, and calculating the probability of neighboring cells having a Ca^{2+} event that was induced within 10 frames after an established Ca^{2+} event occurred in a given cell. An automated computer program developed in the lab marked each cell's position and tracked the cells by calculating each *X* and *Y* centroid location for every registered individual area from the reference frame. To detect these events, the starting frame was chosen manually. Additional scripts were

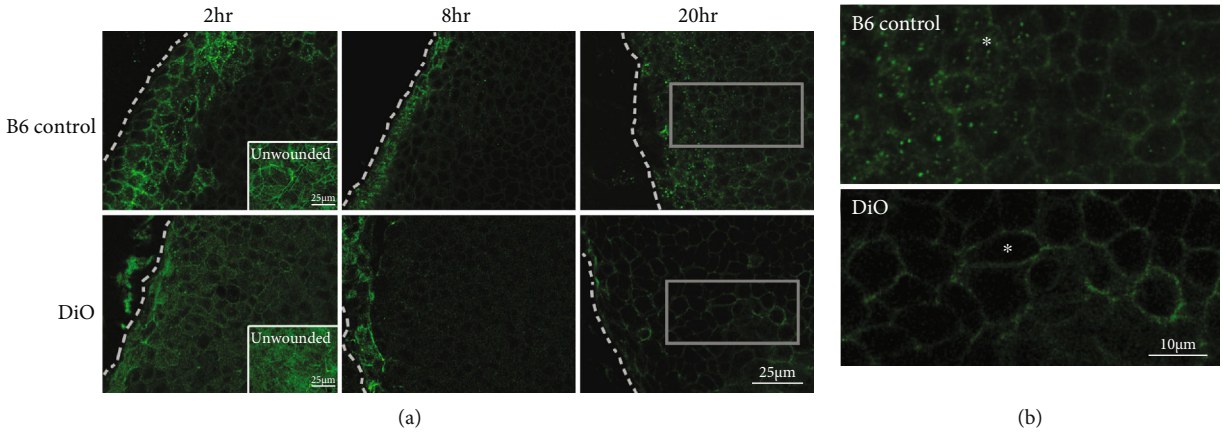

Figure 1: Pannexin1 localization in B6 control and DiO corneal epithelium. (a) Representative confocal immunofluorescent images of pannexin1 in unwounded and wounded corneas: 2, 8, and 20 hrs postepithelial debridement. Dotted line indicates the leading edge. Bar equals 25 microns. (b) Enlarged immunofluorescent images of the rectangular boxes in (a) (20 hrs), which show the cell morphology back from the leading edge. Asterisks (*) mark representative cells in both B6 control and DiO ~6 cells back from the leading edge. Note the elongation of the * cell in DiO. Bar equals 10 microns. *N* is a minimum of 3 independent experiments.

employed to correct for slight movement or drift over the frames.

2.9. Statistical Analysis. Values were obtained by taking the mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical significance was determined by unpaired, one-tailed Student's *t*-test or one or two-way ANOVA with appropriate post hoc tests using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and R studio (RStudio, Inc., Boston, MA).

3. Results and Discussion

3.1. Expression and Localization of Pannexin1. Pannexins belong to a family of proteins that release intracellular ATP through a single membrane channel, which then activate purinoreceptors, specifically the ion channel receptor, P2X7 [24]. The pannexin1 channel has a number of roles in signaling, inflammation, and cytoskeletal reorganization caused by mechanical deformation [22, 23]. Recently, Lee et al. [18] showed in vitro that inhibition of pannexin1 delayed wound healing, and we attributed this to aberrant directional motility. In addition, there was a decrease in cell-cell communication. Previously, we showed that there was a change in localization of P2X7 after an epithelial debridement in B6 control mice. After wound closure and epithelial stratification, this localization returned to that observed in unwounded tissue [17]. In contrast, there was a difference in localization of P2X7 in DiO corneas after wounding [6]. Together, these data led us to speculate that both P2X7 and pannexin1 may act as sensors at the leading edge of a wound; however, in tissue where wound healing is compromised, these sensors may not occur. Furthermore, it is not known if the association between P2X7 and pannexin1 is necessary for cell-cell communication and if this interaction is detected in obese or diabetic tissue.

To further explore this relationship, the localization of pannexin1 was examined in epithelium before and after a debridement wound from B6 control and pretype 2 diabetic (DiO) mice (Figure 1). In both B6 control and DiO unwounded epithelium, pannexin1 was detected along the cell borders (Figure 1(a) (inset)), with the DiO tissue displaying a more extensive cytoplasmic pannexin1 staining. Two hours postdebridement, pannexin1 staining in the B6 control epithelium was intense along the cell borders near the leading edge and about 5-6 rows back (Figure 1(a)). In contrast, pannexin1 in the DiO tissue was diffuse and lacked the intensity detected in the B6 control (Figure 1(a)). At 8 hrs, the area of intense staining along the cell borders in the B6 control tissue decreased to only two rows immediately at the leading edge (dotted line). Interestingly, the staining pattern and intensity in the 8 hr DiO tissue were similar to B6 control. In addition, the leading edge in the DiO tissue was consistently more ragged, as they were more fragile upon manipulation. By 20 hrs, pannexin1 was present along the epithelial cell borders in both DiO and B6 control; however, punctate staining near the leading edge was prominent in the B6 control. We speculate that this localization is indicative of endocytosis in response to elevated ATP. Distal to the leading edge at 20 hrs (Figure 1(a), outline), the prototypical basal epithelial cobblestone shape was present in the B6 control tissue, while the cells were more elongated in the DiO (Figure 1(a), outline; enlarged in Figure 1(b) (asterisk)). We speculate that the elongated morphology was due to a change in mechanical force, which altered the cytoskeletal structure of the cells. This hypothesis was supported by the detection of a similar stretched epithelial phenotype in live-cell movies of cultured epithelial cells treated with 10Panx, a specific inhibitor of pannexin1 [18].

We hypothesize that injury induces a distinct phenotype, and the DiO tissue does not appear to induce the proper signal to activate remodeling. One potential reason for this may be that the compliancy of the diabetic epithelium is reduced causing a change in the mechanical deformation as cells move, which may alter the pannexin1-mediated ATP release and the activation of the P2X7 receptor seen in other tissues

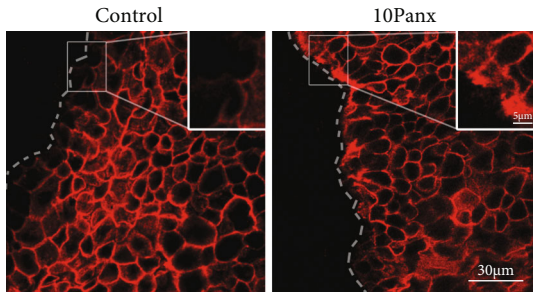

Figure 2: Pannexin1 mediates corneal epithelial sheet migration. Representative images of B6 control corneas (in the presence of 10Panx or a scrambled control (control) peptide) wounded, incubated for 2 hrs in medium, fixed, and then stained with rhodamine phalloidin. The inset highlights the differences in actin at the leading edge and demonstrates coalescence of actin in the 10Panx treated corneas. Bar equals 30 microns. *N* is a minimum of 3 independent experiments.

[23]. Supporting this speculation is experimental data demonstrating that DiO epithelium is significantly softer than the B6 control [8] and preliminary data indicating that diabetic epithelium is softer than DiO tissue.

3.2. Inhibition of Pannexin1 Mediates Actin Reorganization and Cell-Cell Communication. Since the localization of pannexin1 in B6 control and DiO epithelium at 2 hrs postdebridement differed in both intensity and number of cells displaying enhanced staining near the leading edge, we investigated the role that pannexin1 plays in migration and cell-cell communication. To examine this, B6 control corneas were incubated with either 10Panx (a specific peptide to inhibit pannexin1) or a scrambled control peptide and examined 2 hrs after epithelial debridement (Figure 2). When the corneas were incubated in medium containing the scrambled peptide, the F-actin displayed typical actin cytoskeletal reorganization at the leading edge of the wound and was minimal at the side of the cells facing the wound. In the presence of 10Panx, F-actin is coalesced at the leading edge, and there is an irregularity in cell shape near the wound.

To examine the role of pannexin1 in cell-cell communication, Ca^{2+} mobilization live-cell experiments were performed on corneal epithelium after injury. B6 control corneas were loaded with Cal-520 AM, a calcium indicator that has a long half-life and is more intense than Fluo-3 AM, and counter-stained with CellMask Deep Red, a plasma membrane stain that allows for visualization of corneal epithelium [18]. The latter dye allowed us to identify the basal cells while imaging, as the corneal epithelium is multilayered. The corneas were incubated with either the scrambled control peptide (control) or 10Panx, and events were recorded over time (Figure 3(a)). The images were exported into a MATLAB script [18], and MATLAB-detected events were identified as fluorescence events that were greater than a 50% threshold of the maximum normalized fluorescence signal. Cells were identified using coordinates from the image study video, and representative event charts were generated for each condition (Figure 3(b)). When pannexin1 was inhibited, the MATLAB-detected events were dramatically reduced (Figures 3(a) and 3(b)). These results indicate that cell-cell communication requires the pannexin1 channel. In Figure 3(c), we analyzed the role of pannexin1 on communication between cells in the corneal epithelium and demonstrated that there was a significant decrease ($p < 0.05$) in the likelihood (or event probability) that cells would signal one another when pannexin1 was inhibited with 10Panx. These results support our hypothesis that pannexin1 channels play a crucial role in Ca^{2+} mobilization between corneal epithelial cells and that most of the communication occurs at the leading edge. This study allowed us to examine the dynamic communication that occurred after injury in the basal cells at the leading edge. Future experiments will be performed to examine communication between the different layers of the epithelium and regions of the cornea. The coordination of the response observed in Figure 3 may be explained in part by the fact that pannexin1, a channel protein, transports ATP out of the cell and may couple the cells at the leading edge [28]. These data are supported by earlier studies in other cell systems that suggest the presence of a feed-forward system where ATP moves through pannexin1 channels and activates P2X7 receptors [24]. Future studies will examine levels of ATP released when pannexin1 channels are inhibited with 10Panx in obese or diabetic tissue. However, the results from the ex vivo corneal epithelium experiments suggest that the release of ATP along the wound margin was inhibited, thereby removing the ATP gradient that Junger demonstrated in neutrophils [29].

In addition, we wanted to assess if the events that occurred after injury in our ex vivo results were similar to those that we detected from our in vitro experiments. To do this, we compared the probability that cells near the leading edge would communicate to another cell in both systems, and we found that there was no significant difference (Figure 3(d)). The consistency is intriguing, as the in vitro cultures represent a single epithelial layer, while the ex vivo imaging is of basal corneal epithelial cells from an intact cornea.

3.3. Interaction of Pannexin1 and P2X7. The resulting intercellular Ca^{2+} mobilization and actin reorganization of diabetic corneal epithelium indicate an intricate play in communication between the 2 channel proteins, pannexin1 and P2X7. Upon analysis of the proposed pannexin1 signalome [30], we hypothesized that there were significant changes in the interaction between P2X7 and pannexin1 in the pretype 2 diabetic corneal epithelial model (DiO) compared to the B6 control. This change in association may underlie the delayed and disorganized wound healing observed in diabetic corneal models. Although previous immunoprecipitation experiments demonstrated an association between pannexin1 and P2X7 in cultured epithelium [18], these experiments did not address the localization of the associations in unwounded and wounded corneal epithelium, or how the association was altered in a diabetic cornea. To address this, we performed PLA on both unwounded corneas and corneas at 2 hrs after injury. This time point was chosen because previous experiments have shown that there was a significant difference in localization of P2X7 and

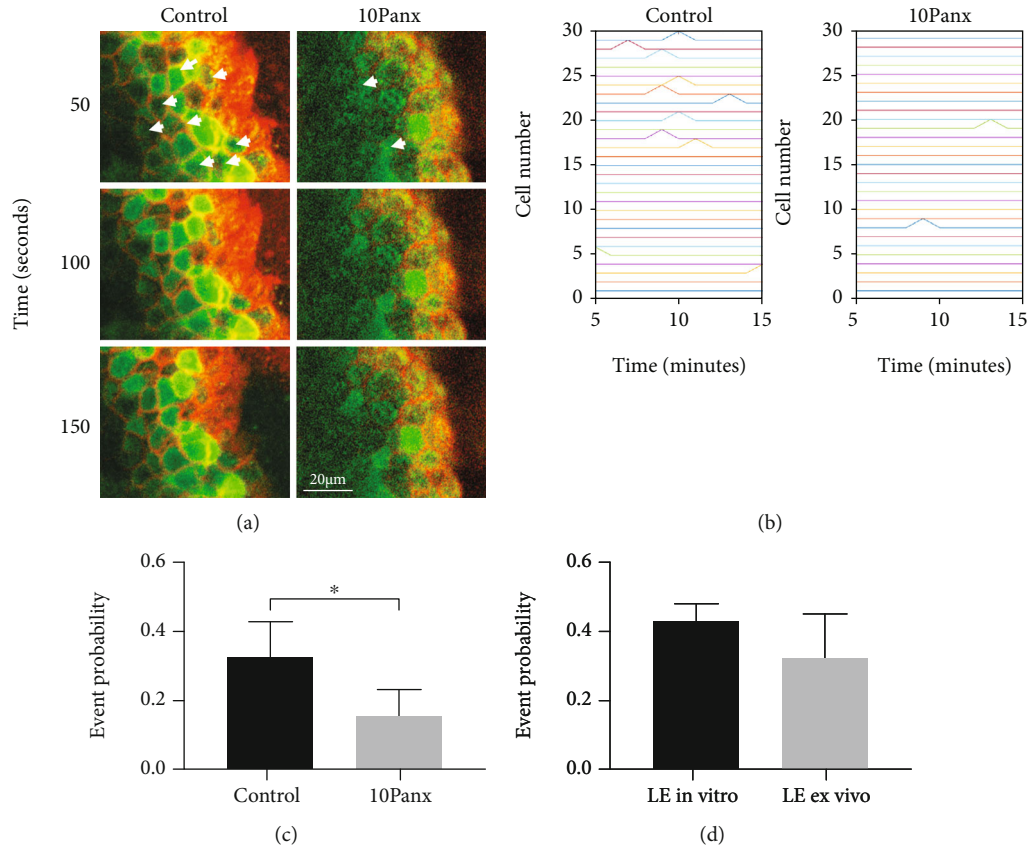

Figure 3: Pannexin1 mediates communication between corneal epithelial cells. (a) Representative images of B6 control corneas incubated in the presence of 10Panx or a scrambled control (control) peptide and then labelled with Cal-520 AM and CellMask Deep Red for imaging. Representative images of the Ca^{2+} wave in basal epithelial cells after injury: 50, 100, and 150 seconds. Arrowheads mark cells to follow over time. (b) MATLAB event charts showing the Ca^{2+} mobilization events detected over time. (c) Event probability values comparing corneas preincubated in the presence of either 10Panx or scrambled control (control) peptide followed by an epithelial scratch injury. (d) Event probability values comparing the wound response near the leading edge of the corneal epithelium ex vivo to in vitro following a scratch wound. Data are expressed as mean ± SEM and were analyzed with a one-way ANOVA with the Tukey's multiple comparison test. $p < 0.05$. N is a minimum of 3 independent experiments.

pannexin1 between the 2 murine models. The amplification process that ultimately generates a visible signal will only occur if the two antibodies are within 40 nm of one another. This technique was used to detect P2X7/pannexin1 interaction throughout the tissue, and specifically, at the region adjacent and distal to the wound.

Optical images were collected from the basal through to the apical layer of the epithelium and presented as enface (basal layer) and orthogonal (full thickness) views (Figure 4(a)). All imaging was performed at the same laser gain and intensity settings that were established through examination of the negative controls and subsequent analysis of overlapping pixel points (see Methods). Counterstaining of the epithelium with rhodamine phalloidin (Figure 4(a), actin) was performed to improve visibility of the tissue. In the unwounded B6 control epithelium, P2X7 and pannexin1 displayed strong colocalization along cell borders (Figure 4(a)). In the orthogonal image, there were many puncta in both the apical and basal cells. In the DiO enface and orthogonal sections, the P2X7 and pannexin1 association was similar to the B6 control (Figure 4(a)). Two hours after injury, there was a distinct change between the association in B6 control and DiO tissue. In the B6 control, there was intense staining in the enface and orthogonal images. There were significantly more PLA punctae in most apical cells than there were in the basal cells, and there appeared to be diminished association at the leading edge (Figure 4(a), ortho). In the DiO tissue, after injury, there was a "line" of fluorescence at the leading edge, yet substantially fewer colocalized punctae back from the wound. As with the B6 controls, the DiO corneas displayed intense staining of apical epithelial cells; however, there was only minimal association in the basal cells. This qualitative data was exported into ImageJ for preliminary processing and then analyzed and quantified using the CellProfiler software. We optimized the CellProfiler pipeline to identify and enhance punctae of the appropriate size, as determined by preliminary analysis in ImageJ. Cells were analyzed individually to ensure optimal detection of PLA puncta. Several cells both at and back from the leading edge of the wound were

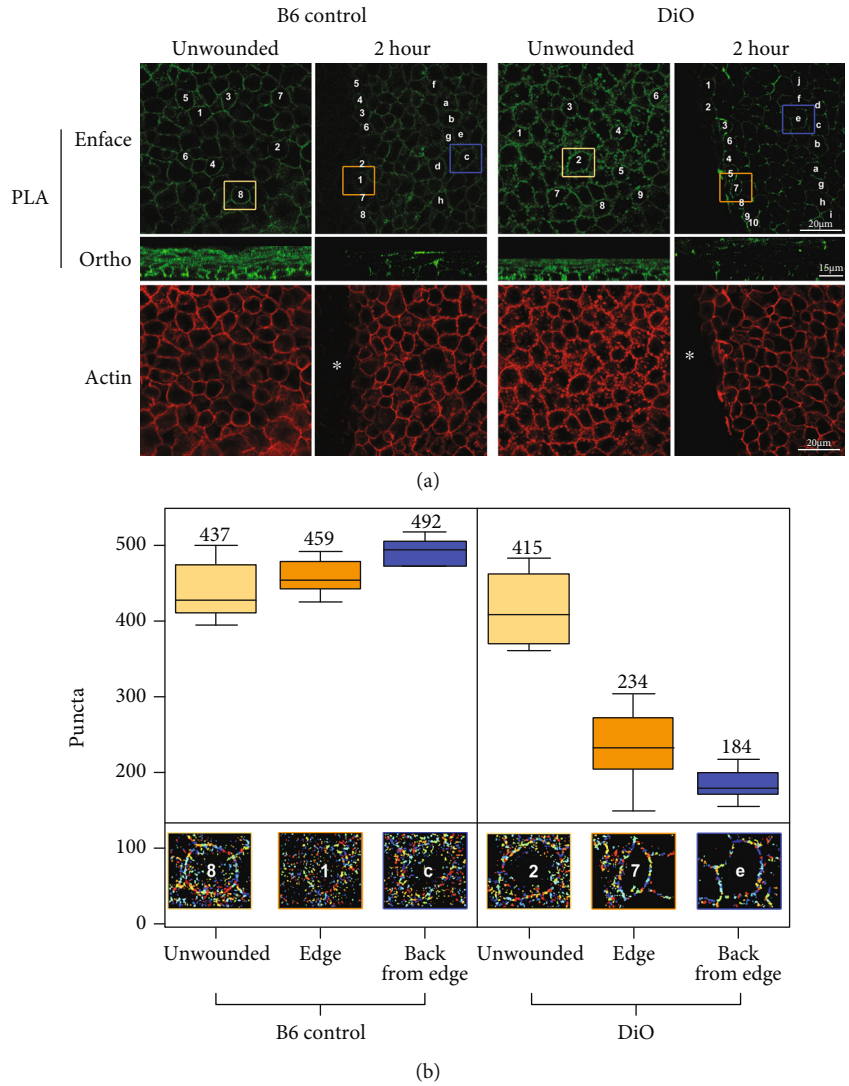

Figure 4: Association of pannexin1 and P2X7 in unwounded corneas and 2 hrs after injury in DiO and B6 control corneal epithelium. Proximity ligation assays were performed using antibodies to P2X7 and pannexin1. (a) Representative enface and orthogonal (ortho) images of B6 control and DiO corneas are shown. The green color displays puncta of association. Corneal epithelium was counter stained with rhodamine phalloidin (asterisk marks wound area). The numbers and letters mark the individual cells that were analyzed using CellProfiler near the wound and back from the wound. The boxes indicate the representative individual cells that are shown in (b) after PLA analysis with CellProfiler. Bar equals 20 microns (PLA enface and actin) or 15 microns (PLA ortho). (b) Analysis of overlapping puncta for each condition (unwounded, leading edge, back from leading edge) was performed and a box plot drawn using R. The mean puncta for each region are placed above the box. Mean ± SEM are plotted, and two-way ANOVA with Tukey's multiple comparison of means was performed. $p < 0.05$. N is a minimum of 3 independent experiments.

chosen for analysis to ensure statistical significance. The mean number of puncta for unwounded, edge of wound, and back from edge of wound conditions was calculated for both B6 control and DiO samples. Two-way ANOVA with Tukey's multiple comparison of means was performed. In the unwounded tissue, there was no statistically significant difference in the number of puncta per micron between B6 control and DiO tissue ($p = 0.779$) (Figure 4(b)). Furthermore, after injury in the B6 control, the number of puncta did not change significantly ($p = 0.792$ for the edge and $p = 0.0238$ back from the edge). In contrast, in the DiO tissue, there was a significant decrease in puncta both near and distal to the leading edge ($p = 0.0000001$ for both) (Figure 4(b)). We predict that the lack of association may be an underlying reason for the aberrant migration in DiO corneas after wounding and have preliminary data that the diabetic cells migrate, but the directionality is impaired. In the future, Ca^{2+} mobilization and migration experiments will be performed on diabetic corneal epithelium to determine the downstream effects of decreased cell-cell communication.

These are the first studies to use quantitative image processing of tissues to examine cell-cell communication and to correlate this data with the association of 2 ion channels. This model may be used to identify therapeutic targets and test strategies in the cornea and in other tissues to modulate the treatment and prevent disease progression.

4. Conclusions

In summary, our study provides comprehensive data demonstrating that pannexin1 has a different localization in B6 control and an obese pretype 2 diabetic model (DiO). In addition, pannexin1 inhibition impairs cell-cell communication of corneal epithelium, indicating that pannexin1 plays a critical role in wound repair. In the pretype 2 diabetic corneal epithelium, the association of P2X7 and pannexin1 was negligible, indicating that the interaction of the proteins was lost, thereby, disrupting the interactome and subsequent downstream signaling.

Data Availability

The immunohistochemical and signaling data used to support the findings of this study are included within the article. Previously reported Ca^{2+} mobilization data was used to support this study and is available at doi: 10.1371/journal.-pone.0213422. These prior studies and datasets are cited at relevant places within the text and referenced as Lee et al. [18].

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Cellular Contractility Profiles of Human Diabetic Corneal Stromal Cells

Thi N. Lam,[1] Sarah E. Nicholas,[2,3] Alexander Choi,[1] Jian-Xing Ma,[4,5] and Dimitrios Karamichos[2,3,6]

[1]*Dean McGee Eye Institute, Oklahoma University Health Sciences Center, 608 Stanton L Young Blvd, Oklahoma City, OK 73104, USA*
[2]*North Texas Eye Research Institute, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107, USA*
[3]*Department of Pharmaceutical Sciences, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107, USA*
[4]*Department of Physiology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young, Oklahoma City, OK, USA*
[5]*Harold Hamm Oklahoma Diabetes Center, 1000 N Lincoln Blvd, Oklahoma City, OK, USA*
[6]*Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107, USA*

Correspondence should be addressed to Dimitrios Karamichos; dimitrios.karamichos@unthsc.edu

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Diabetic keratopathy is a corneal complication of diabetes mellitus (DM). Patients with diabetic keratopathy are prone to developing corneal haze, scarring, recurrent erosions, and significant wound healing defects/delays. The purpose of this study was to determine the contractility profiles in the diabetic human corneal stromal cells and characterize their molecular signatures. Primary human corneal fibroblasts from healthy, Type 1 DM (T1DM), and Type 2 DM (T2DM) donors were cultured using an established 3D collagen gel model. We tracked, measured, and quantified the contractile footprint over 9 days and quantified the modulation of specific corneal/diabetes markers in the conditional media and cell lysates using western blot analysis. Human corneal fibroblasts (HCFs) exhibited delayed and decreased contractility compared to that from T1DMs and T2DMs. Compared to HCFs, T2DMs demonstrated an initial downregulation of collagen I (day 3), followed by a significant upregulation by day 9. Collagen V was significantly upregulated in both T1DMs and T2DMs based on basal secretion, when compared to HCFs. Cell lysates were upregulated in the myofibroblast-associated marker, α-smooth muscle actin, in T2DMs on day 9, corresponding to the significant increase in contractility rate observed at the same time point. Furthermore, our data demonstrated a significant upregulation in IGF-1 expression in T2DMs, when compared to HCFs and T1DMs, at day 9. T1DMs demonstrated significant downregulation of IGF-1 expression, when compared to HCFs. Overall, both T1DMs and T2DMs exhibited increased contractility associated with fibrotic phenotypes. These findings, and future studies, may contribute to better understanding of the pathobiology of diabetic keratopathy and ultimately the development of new therapeutic approaches.

1. Introduction

Diabetes mellitus (DM) is a major public health problem and one of the most prevalent chronic diseases worldwide [1]. DM continues to rise in numbers and significance, affecting epidemic proportions globally [2]. In 2014, the World Health Organization (WHO) stated that approximately 422 million adults worldwide were suffering from DM, almost doubling from 4.7% in 1980 to 8.5%. The number of people with DM is projected to further increase since the disease is not only predominate in adults but children as well [3–5].

The etiology of the two most common DM types, Type 1 and Type 2, is a complex interplay of genetics, lifestyle preferences, and environmental factors [6]. Type 1 DM

(T1DM) is a consequence of autoimmune beta cell destruction, which leads to insulin deficiency, and accounts for approximately 10% of cases, primarily children and young adults. On the other hand, Type 2 DM (T2DM) accounts for 90% of cases and generally forms part of a metabolic interaction, which is defined by insulin resistance, cardiovascular risk factors, and obesity [7, 8]. DM-related ocular complications are one of the leading causes of adult blindness [9, 10]. Both diabetic retinopathy and diabetic keratopathy are eminent risk factors for visual deterioration in DM patients, resulting in more than 20,000 new cases of blindness every year [11, 12].

Diabetic keratopathy-related abnormalities may include, but are not limited to, stromal edema, deposition of advanced glycation end products, decreased corneal sensitivity, recurrent corneal erosions, delayed corneal wound healing, and neurotrophic corneal ulcers [13–15]. When the corneal epithelium and stromal layers are affected, it is vital that the stroma and epithelium reattach or further tissue defects can develop which often reoccur. Furthermore, under hyperglycemic conditions, increased reactive oxygen species (ROS), advanced glycation end product (AGE) immune reactivity, and mitochondrial injury in the cornea have all been reported [16]. The diabetic corneal epithelium and stroma are resistant to traditional treatment regimens, due to the fact that hyperglycemia significantly changes the structure and function of both layers, resulting in altered levels of cell proliferation, weak barrier function, abnormal collagen deposition, and collagen crosslinking [17–21]. Such alterations expose diabetic patients to higher susceptibility of corneal infections, stromal ulcerations, erosion, scarring, and ultimately vision loss [22].

The human corneal stroma is rich in type I collagen, but also contains type V and type VI collagens [23, 24]. The cornea harbors dormant keratocytes that, when activated by pathological processes, can differentiate into active fibroblasts and subsequently myofibroblasts which deposit type III collagen. Type III collagen is critical upon injury/trauma and the subsequent wound healing cascade. This transformation process is critical to corneal wound closure and contraction. Unfortunately, myofibroblasts are responsible for the pathologic processes of corneal haze and scarring [25–27].

In the present study, we utilized a detached, free-floating 3D collagen gel model, in order to determine the contractile "signatures" of the diabetic corneal stroma cells [28]. We further investigated the modulation of established corneal and diabetic markers as a function of contraction. Overall, our study highlights the importance of utilizing 3D collagen gels to examine cellular-extracellular matrix (ECM) interactions in the context of diabetic keratopathy. To our knowledge, this is the first study utilizing 3D free-floating collagen gels to investigate the diabetic corneal stroma.

2. Materials and Methods

2.1. Ethics and Inclusion Criteria. All parts of this study adhere to the Declaration of Helsinki ethical principles. Institutional review board approval was received prior to initiation of all experiments defined in this study. Human corneal samples were obtained from the National Development and Research Institute (NDRI) and the Oklahoma Lions Eye Bank. The North Texas Regional Institutional Review Board (IRB) was notified, and appropriate permission was obtained prior to initiations of experimental procedures (#2020-30). All diabetic samples adhered to strict inclusion/exclusion criteria where DM donors with a clinical diagnosis of T1DM or T2DM were included, only if no other systemic and unrelated diseases or ocular pathology existed. The healthy control group was comprised of corneas isolated from cadavers with no history of ocular trauma or systemic diseases. The cause of death for healthy controls ranged from accidental to non-DM-related diseases (blunt force trauma, head trauma, end-stage renal disease, acute segment elevation myocardial infarction, subarachnoid hemorrhage, and cardiac arrest). In this study, a total of 8 diabetic donor corneal samples (4 donors for each T1DM and T2DM) and 4 healthy age-matched control samples were analyzed. The average age range for donors included in this study was 55-59 years of age, and the duration of DM was from 3 to 30 years. We observed no data bias, based on the age or duration of DM.

2.2. Primary Cell Isolation and Cultures. Healthy and DM corneas were obtained and processed. Stromal cells were isolated as previously described [29, 30]. Briefly, both the endothelium and epithelium were removed from the stroma by scraping briefly with a razor blade; furthermore, the stroma was cut into $\sim 2\,\text{mm} \times 2\,\text{mm}$ pieces. The corneal pieces were then allowed to adhere to the bottom of a T75 flask for 30 minutes at 37°C before adding 10% Fetal Bovine Serum (FBS) Eagle's Minimum Essential Media (EMEM) and 1% antibiotic/antimycotic (Gibco® Antibiotic-Antimycotic, Life Technologies). At approximately 80% confluency, the explants were passaged in 10% FBS in EMEM and 1% antibiotic for further expansion. All experiments were executed using cells between passages 3 and 7.

2.3. Collagen Contraction Assay. Rat-tail collagen type I (Advanced Biomatrix, San Diego, California) was mixed with EMEM on ice with 125 μL EMEM per 1 mL collagen. The pH was then adjusted to pH 7–8 with 1 M NaOH. Healthy human corneal fibroblasts (HCFs), T1DMs, or T2DMs were added at a concentration of 5×10^5 and mixed slowly to avoid air bubbles. This mixture was plated in a 12-well plate at 1 mL per well and incubated in 37°C for 30 min to promote solidification. After congealing, 1 mL of 10% FBS EMEM was added on top of the gels (Karamichos et al., 2009; Lyon et al., 2015). The collagen gels were released after 48 h of incubation by running a sterile blade around the edges of the well. The contraction of the collagen gels was monitored by measuring the gel diameter daily for 9 days. The area of the gel was quantified using ImageJ software. Calculating the contractility rate, the average area of the gels was subtracted from the average area on day 0 and divided by the number of days that had passed.

2.4. Western Blot Analysis. Western blots were performed on cell lysates and conditional media collected from all

experiments, as per our previously optimized protocol [31]. Preparation of cell lysates was initiated by using RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing protease and phosphatase inhibitors (Sigma Aldrich; St. Louis, MO), followed by brief incubation and centrifugation and stored at -20°C until further processing. Total protein content within conditioned media and cell lysates was measured using a BCA assay (Thermo Scientific, Rockford, IL, USA). Samples were then normalized to the sample containing the lowest protein content, thereby enabling equal loading onto the gel. Media samples were run on a 4–20% precast polyacrylamide gradient gel at 130 V for 1.5 h then transferred to a nitrocellulose membrane on ice at 100 V for 1 h. The membrane was blocked in a 5% milk solution in Tris-buffered solution with Tween 20 (TBST) for 1 h, followed by overnight incubation in a cold room with 1 : 1000 primary antibodies. Antibodies used include collagen I (ab34710; Abcam, Cambridge, MA, USA), collagen III (ab7778; Abcam, Cambridge, MA), collagen V (ab94673; Abcam, Cambridge, MA), α-SMA (ab5694; Abcam, Cambridge, MA), IGF-1 (Abcam; Cambridge, MA), IGF-1R (Abcam; Cambridge, MA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab9485; Abcam, Cambridge, MA). After primary incubation, the membrane was washed for 5 min (3x) in TBST before probing with secondary antibody Goat anti-Rb Alexa Fluor 568 (Life Technologies, Grand Island, NY, USA) at room temperature for 1 h with rocking. The membrane was allowed to dry before imaging using ChemiDoc-It to image. Western blots were quantified using densitometry utilizing pixels measured within each band.

2.5. Statistical Analysis. Statistical analyses were carried out using a 2-way ANOVA and Welch's unpaired t-test, calculated by GraphPad Prism 6 software. $p < 0.05$ and lower ($p < 0.01$, $p < 0.001$, etc.) were considered statistically significant. Error bars represent standard deviation. Data is representative of at least three independent experiments per donor.

3. Results

3.1. Contraction Profiles. HCFs embedded in collagen gels maintained an average of 166.4 mm^2 of their matrix area by day 9. On the other hand, the gel matrix area seeded with T1DMs and T2DMs only maintained an average of 136.7 mm^2 and 48.9 mm^2, respectively. At day 1, we identified an average of 69.3 mm^2 reduction in gel area by HCF controls compared to a reduction of 109.9 mm^2 in matrix area in T1DMs and 211.7 mm^2 in T2DMs (Figure 1). By day 3, HCFs had contracted their matrix at an average rate of 26.08 mm^2/day compared to a contraction rate of 40.97 mm^2/day and 76.00 mm^2/day by T1DMs and T2DMs, respectively. Our results suggest that both T1DMs and T2DMs display significantly accelerated contractility ($p < 0.0001$).

3.2. Corneal Fibrotic Markers

3.2.1. Collagen Assembly. The corneal stroma is composed of various collagen types such as Col I and V, but with diseases like diabetic keratopathy, the corneal stroma composition

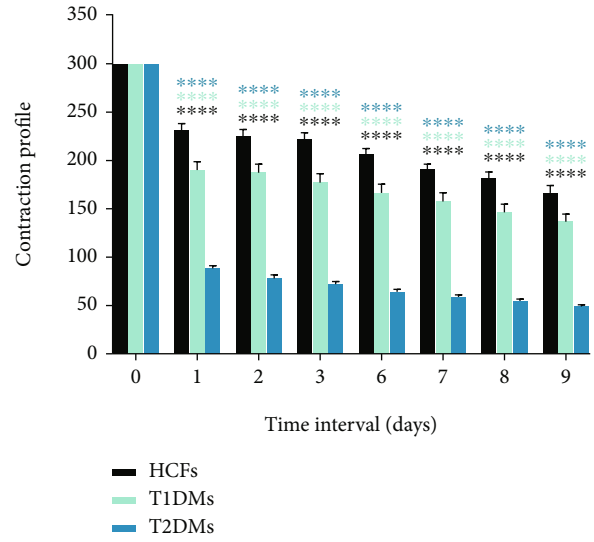

Figure 1: Quantification of contraction collagen matrix in HCFs, T1DMs, and T2DMs from days 0 to 9. A significant reduction is observed in the area of the collagen matrix between the three cell types correlating with increased contractility. Two-way ANOVA ($n = 12$) was used to analyze the results. Error bars represent standard deviation (**** denotes $p < 0.0001$).

can alter significantly. We examined protein expression in HCF, T1DM, and T2DM lysates. Initially, at day 3, there were no significant differences of Col I expression in T1DMs or T2DMs, compared to HCFs (Figure 2(a)). However, at day 9, both T1DMs and T2DMs showed significant upregulation ($p < 0.05$, $p < 0.01$), when compared to HCFs. T1DM and T2DM also showed increased expression ($p < 0.01$, $p < 0.05$) of Col I at day 9. No significant modulation of Col III expression was observed (Figure 2(b)). T2DMs showed significant upregulation ($p < 0.01$, $p < 0.05$) in both Col V and α-SMA, at day 9, when compared to HCFs (Figures 2(c) and 2(d)). Data analysis on both Col I/Col III and Col I/Col V ratios (Figure 3) revealed no significant changes among different cell types.

3.2.2. Collagen Secretion. We measured Col I, Col III, and Col V secreted into the media by HCFs, T1DMs, and T2DMs, as a function of contraction progress. Col I levels were significantly decreased ($p < 0.05$) in T2DMs at day 3, but not at day 9, when compared to HCFs (Figure 4(a)). We found a trend of upregulated Col III on T1DMs at day 9 and T2DMs at day 3; however, significance was not reached (Figure 4(b)). Col V secretion on day 3 of both T1DMs and T2DMs showed significant upregulation ($p < 0.001$, $p < 0.05$) compared to HCFs, followed by significant downregulation ($p < 0.0001$, $p < 0.05$) on day 9 (Figure 4(c)).

Col I/Col III and Col I/Col V secretion ratios were also analyzed. There were no significant differences in the secretion ratio of Col I/Col III between the diabetic cells and healthy cells (Figure 5(a)). However, T1DMs showed a significant decrease ($p < 0.01$) of secretion between day 3 and day 9. Col I/Col V secretion was significantly downregulated ($p < 0.001$) for T1DMs and T2DMs, at both days 3 and 9, when compared to the HCFs (Figure 5(b)).

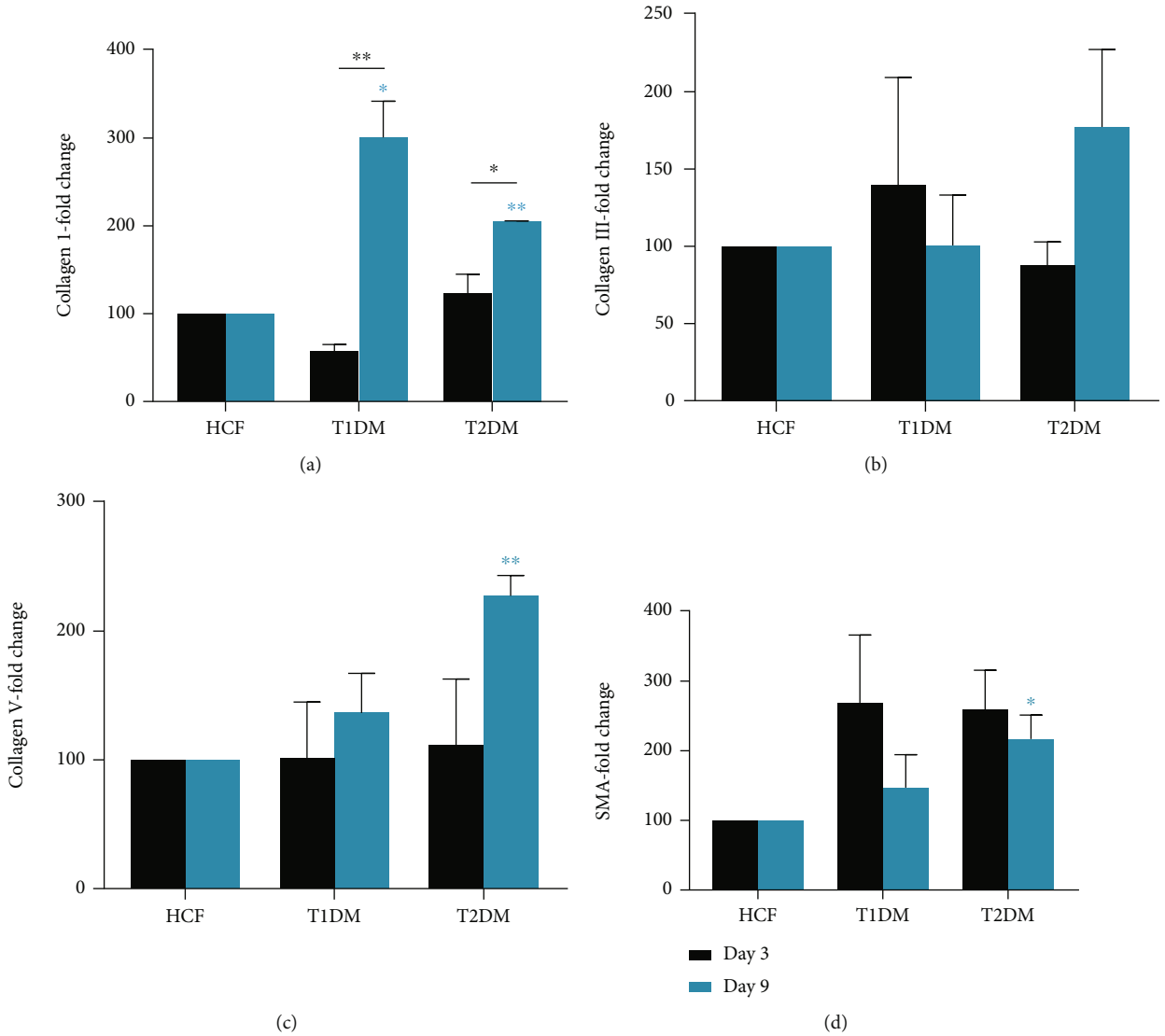

Figure 2: (a) Collagen I (Col I), (b) collagen III (Col III), (c) collagen V (Col V), and (d) α-SMA (SMA) protein levels in HCF, T1DM, and T2DM on days 3 and 9. Col I was significantly increased on day 9 in the T1DM and T2DM compared to the HCFs and between days 3 and 9. Significant increases in Col V and SMA were seen at day 9 for the T2DM when compared to HCFs at day 9. Welch's unpaired t-test and/or ordinary one-way ANOVA ($n = 4$) was used to analyze the results. Error bars represent standard deviation ($**$ denotes $p < 0.01$ and $*$ denotes $p < 0.05$).

3.2.3. IGF-1 and IGF-1R Protein Expression by HCFs, T1DM, and T2DM. We determined modulation of the key mediators in DM, insulin-like growth factor 1 (IGF-1) and its receptor (IGF-1R). Our data revealed significant upregulation ($p < 0.01$) in IGF-1 protein expression in T2DMs when compared to HCFs on day 3 (Figure 6(a)). However, IGF-1 expression was significantly downregulated at day 3 in T1DMs, including a significant decrease on day 9, indicating a possible interplay between collagen I contraction phenotype and IGF-1 DM mediator mainly in T2DM cells. IGF-1R revealed no significant changes between days 3 and 9 in HCFs, T1DMs, or T2DMs (Figure 6(b)). These results suggest that IGF-1 and IGF-1R activity might correlate with the altered contractile state of the diabetic corneal stroma.

4. Discussion

Diabetic keratopathy is a degenerative corneal disease observed in patients suffering from systemic DM. About 46-64% of DM patients are at risk to develop diabetic keratopathy, and with about 1 million T1DM patients in the US, diabetic keratopathy is a serious vision-threatening condition [32]. Various causes of diabetic keratopathy have been proposed, including structural abnormalities in the corneal epithelium basement membrane [33]. Studies suggested that corneal stroma, with altered/damaged basement membrane, is the reason for a delay in corneal epithelial wound healing [34, 35]. These structural changes of the basement membrane in the diabetic cornea may account for the loose attachment of corneal epithelial cells.

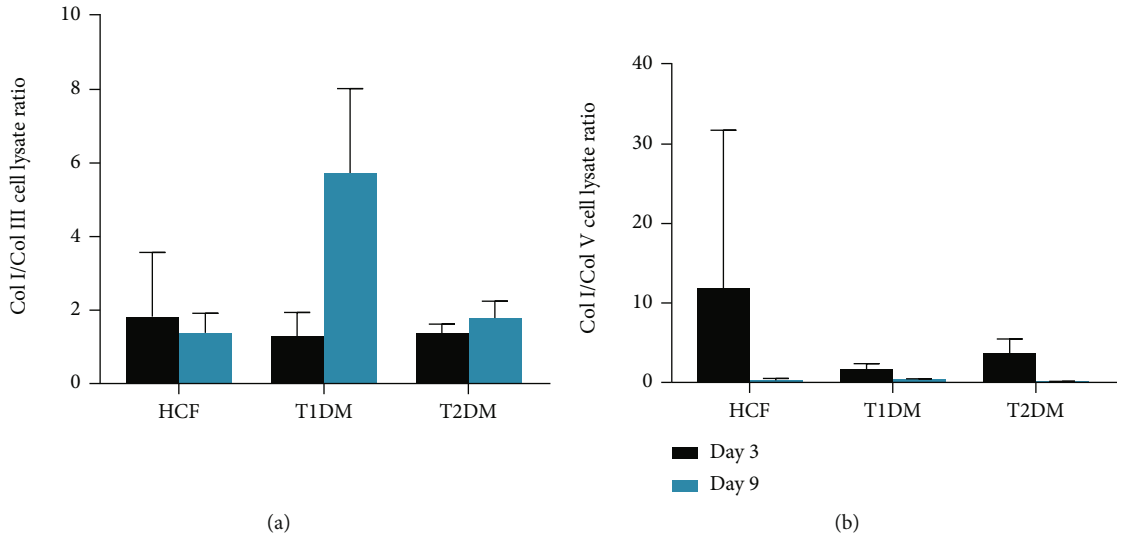

Figure 3: Lysate ratios of (a) Col I/Col III and (b) Col I/Col V. Welch's unpaired t-test and/or ordinary one-way ANOVA ($n = 4$) was used to analyze the results. Error bars represent standard deviation.

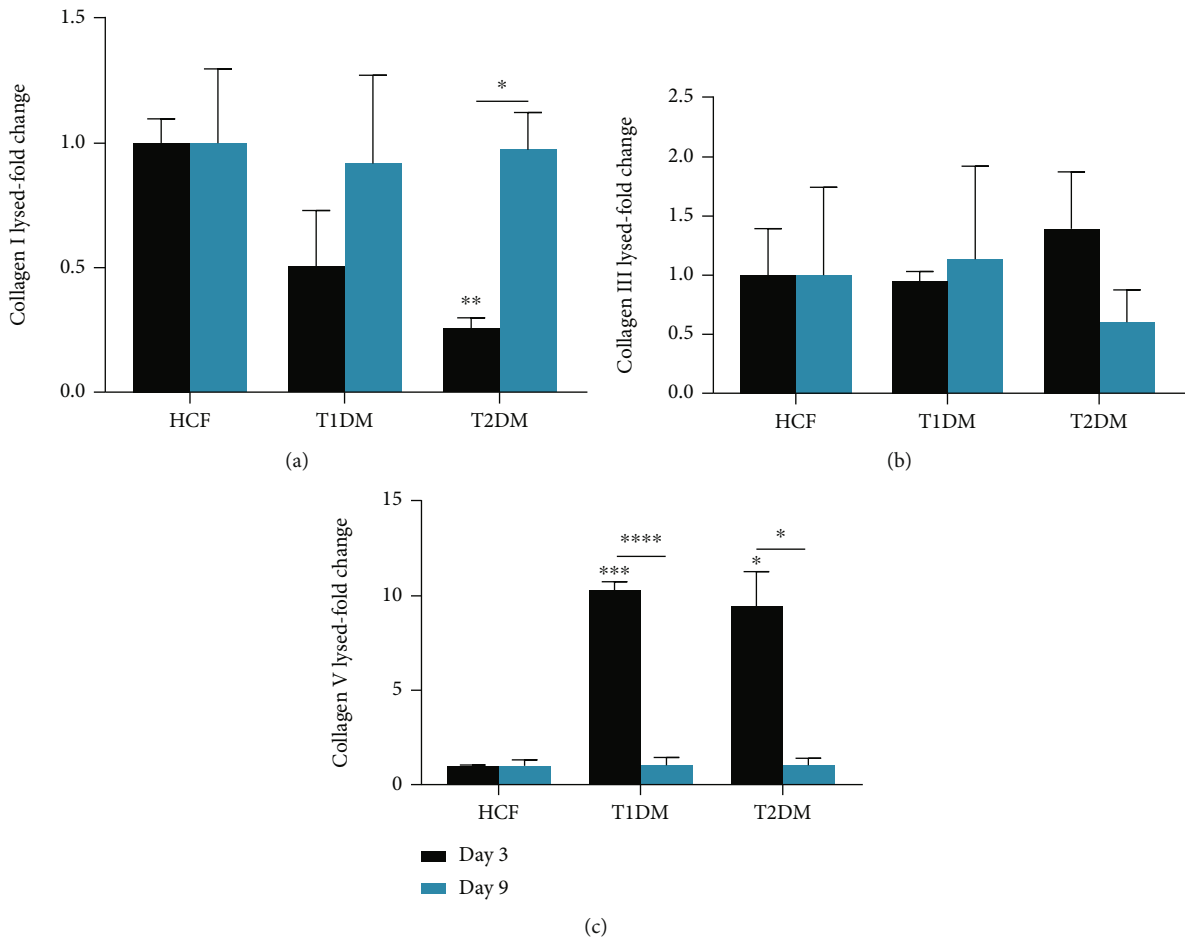

Figure 4: (a) Collagen I (Col I), (b) collagen III (Col III), and (c) collagen V (Col V) protein levels in conditioned media of HCF, T1DM, and T2DM on days 3 and 9. Col I expression significantly decreased on day 3 but not at day 9 compared to the HCFs. T1DM and T2DM show significant increases in Col V expression, followed by significant decreases of secretion at day 9. Welch's unpaired t-test and/or ordinary one-way ANOVA ($n = 4$) was used to analyze the results. Error bars represent standard deviation ($****$ denotes $p < 0.0001$, $***$ denotes $p < 0.001$, $**$ denotes $p < 0.01$, and $*$ denotes $p < 0.05$).

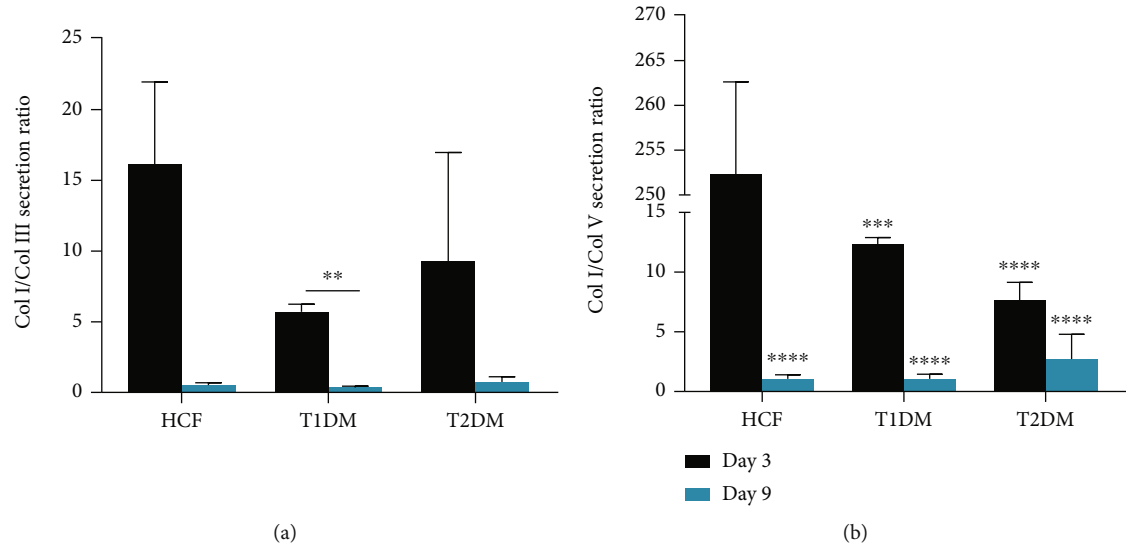

Figure 5: Collagen secretion ratio of (a) Col I/Col III and (b) Col I/Col V. T1DMs had a significant difference between its day 3 and 9 Col I/Col III ratio. For the Col I/Col V ratio, both days of the T1DM and T2DM showed significant decreases of secretion, as well as the HCFs at day 9. Welch's unpaired t-test and/or ordinary one-way ANOVA ($n = 4$) was used to analyze the results. Error bars represent standard deviation (**** denotes $p < 0.0001$ and ** denotes $p < 0.01$).

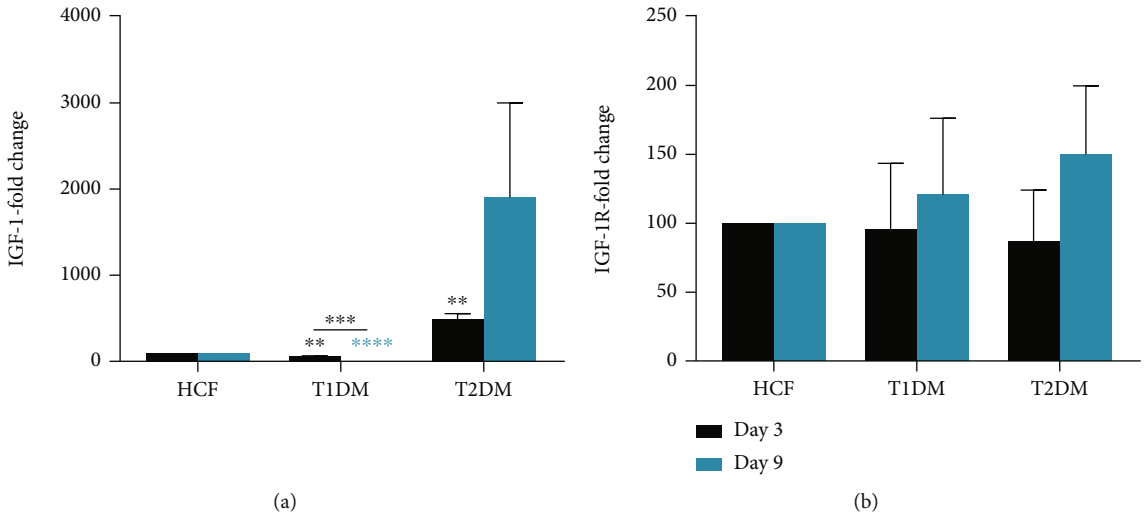

Figure 6: (a) Insulin-like growth factor 1 (IGF-1) and (b) insulin-like growth factor 1 receptor (IGF-1R) protein expression in HCF, T1DM, and T2DM on days 3 and 9. Welch's unpaired t-test ($n = 4$) was used to analyze the results. Error bars represent the standard deviation (**** denotes $p < 0.0001$, *** denotes $p < 0.001$, and ** denotes $p < 0.01$).

The corneal epithelial abnormalities in DM patients have been reported in both human and animal models. In a DM rat model of wound healing, the corneal epithelial wound closure was delayed and the phenotype of epithelium was changed [36, 37]. In addition, abnormal changes of epithelium in DM patients after cataract surgery were noted with increased average cell area and decreased hemidesmosomes. A study by Schultz et al. [13] demonstrated that corneal epithelial lesions, ranging from superficial punctate keratitis to full thickness, break in up to two-thirds of DM patients in their study [38]. The authors also reported a correlation between the severity of keratopathy and the patients' diminished peripheral sensation, suggesting that their epithelial defects and reduced corneal sensitivity are believed to be symptoms of the generalized polyneuropathy that occurs in these patients [39]. Reduced corneal sensitivity predisposes patients to corneal trauma, puts them at greater risk of developing neurotrophic corneal ulcers [40], and adversely affects corneal wound healing [41, 42].

Interestingly, high glucose was shown to independently suppress the epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt signaling pathway and altered corneal epithelial wound healing *in vitro* [43]. In addition, decreased corneal sensation and loss of specific nerve factors have been

proposed as causative players in the development of diabetic keratopathy. Nakamura et al. have revealed that insulin-like growth factor 1 (IGF-1) and substance P, a neuropeptide present in sensory nerves, accelerate corneal epithelial wound healing [44]. In addition, the authors displayed that topical application of substance P and IGF-1 accelerated the corneal epithelial wound healing process in DM animals. These studies help to strengthen the potential pathogenic link between decreased corneal sensation and diabetic keratopathy. Studies by He et al. [45] revealed an entire view of the nerve architecture in human diabetic corneas. They found that decreased epithelial nerve density may result from the abnormalities of stromal nerve architecture and is affected by >5 years of T1DM. These alterations in the stromal nerves can explain the poor healing and persistent epithelial defects seen in DM patients.

The corneal stroma is also known to be affected, in diabetic keratopathy, although studies are lacking [46, 47]. The process of stromal wound healing involves wound contracture, bringing the margins of open wounds together [48]. Mechanical forces generated by fibroblast cells lead to wound contraction and potentially tissue scarring. The response of corneal keratocytes to growth factors secreted postinjury can also be modulated by changes in ECM stiffness. Several studies [49] have demonstrated that fibroblast growth factor-2 (FGF2) induces fibroblast transformation of keratocytes on rigid 2D substrates, as shown by changes in cell morphology and development of stress fibers and focal adhesions [50]. However, within hydrated 3D collagen matrices, FGF2 stimulates ruffling of keratocyte processes without inducing major changes in cell morphology, formation of stress fibers, or collagen matrix organization [51]. When corneal keratocytes are seeded within compressed 3D collagen matrices, fibroblast transformation is again observed [52]. Various studies are aimed at understanding the mechanisms involved in tissue contraction after corneal stromal wounding; Hibino et al. showed rabbit keratocytes cultured in a collagen gel, which contracted in the presence of Fetal Calf Serum (FCS) [53]. These findings highlight the fact that in corneal wound healing, the epithelial cells and keratocytes modulate cellular activities facilitating epithelial migration. In addition, Andresen et al. also utilized collagen gels to demonstrate that the composition of the ECM influences the motility of the human corneal fibroblasts, either through cell-matrix or matrix-matrix interactions, thus facilitating entry of migrating keratocytes into the wound [54].

In this study, we assessed the diabetic corneal fibroblast behavior utilizing 3D collagen matrices. DM corneal cells demonstrated structural interactions with collagen fibrils, resulting in contraction of the gel matrices. By utilizing preassembled 3D collagen gels seeded with HCFs, T1DM, and T2DM cells, changes in contraction rate were measured over a period of 9 days and compared to healthy controls. DM cells showed increased contractility correlated with specific corneal fibrotic markers.

Assessment of overall ECM contraction is a valuable assay for assessing DM changes in cell contractility compared to HCFs. Our data revealed that mainly T2DM and to a lesser extent T1DM display a significantly accelerated contractility of the collagen gel matrix and altered collagen I and V expression compared to HCFs. Contraction of the ECM is important in normal wound healing processes within the cornea. The accelerated contraction profile exhibited by DM cells suggests that stromal fibroblasts in DM corneas are responding to external stimuli, perhaps overreacting leading to more scarring/fibrosis. Depending on the balance of DM mediators, the extent of the wound, and the duration of a hyperglycemic state, the outcome of stromal healing can be regeneration of normal stromal structure or an opaque scar. Our collagen gel model mirrors an *in vivo* DM corneal stroma and allows the dissection of the stromal environment and its resident cells during ECM remodelling and wound repair. Thus, our 3D collagen gel model allowed us to simulate a stromal *in vivo* environment and study the stromal wound healing process.

5. Conclusions

Our data supports DM keratopathy as a stressor of ECM remodelling generating large contractile forces, both of which can alter corneal clarity and result in corneal scarring. In human DM cells, our study revealed abnormal overexpression of insulin-like growth factor (IGF) 1 and suggests that diabetic keratopathy is a result of decreased migratory growth factor levels that could lead to BM degradation and clinically observed delayed wound healing compared with normal cells. The finding confirms that the mechanical properties of collagen matrices should be considered in the cellular contraction events induced by DM keratopathy. Overall, the study has shown the importance of matrix properties in the design of collagen-based biomaterial for clinical applications.

Data Availability

Data will be available upon request from the corresponding author. Please contact Dr. D. Karamichos (dimitrios.karamichos@unthsc.edu).

Conflicts of Interest

The corresponding author is the chief editor of ACP. All other authors have no conflicts of interest.

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