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Unravelling the stromal-nerve interactions in the human diabetic cornea

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Abstract

Corneal defects due to diabetes mellitus (DM) may cause severe vision impairments. Current studies focus on the corneal epithelium and nerve defects neglecting the corneal stroma. The aim of this study was to develop a 3D *in vitro* model to examine the interactions between corneal stroma and nerves in the context of DM. Primary human corneal stromal fibroblasts isolated from healthy (HCFs), Type 1 (T1DM) and Type 2 (T2DM) patients were stimulated with stable ascorbic acid to secrete and assemble an extracellular matrix (ECM). Human neuronal cells were then seeded on top and differentiated to create the 3D co-cultures. Our data revealed successful co-culture of stromal fibroblasts and neuronal cells with large elongated neuron extensions. T2DM showed significant upregulation of Collagen III and IGF1 when compared to T1DM. Interestingly, upon nerve addition, those markers returned to HCF levels. Neuronal markers were also differentially modulated with T2DM co-cultures expressing high levels of β III tubulin where T1DM co-cultures expressed Substance P. Overall, our unique 3D co-culture model provides us with a tool that can be utilized for both molecular and therapeutic studies for diabetic keratopathy.

Keywords

Diabetes mellitus; Diabetic corneal stroma; Corneal nerves; 3D *in vitro* model

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Authors' contributions

S.P. conducted experiments, analyzed data, and wrote the manuscript. T.G.R. conducted experiments and reviewed/edited the manuscript. D.K. conceived the project, provided reagents and supplies, and reviewed/edited the manuscript. J.XM reviewed/edited the manuscript.

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1. Introduction

Diabetes mellitus (DM) is primarily characterized by an elevated blood glucose level, and it is the most common metabolic disorder of the present times. (Schultz et al., 1981; Schultz et al., 1983; Priyadarsini et al., 2016a,b). Prevalence of DM has increased over the years and is expected to affect over 500 million people worldwide by 2030 (Priyadarsini et al., 2016a,b). DM presents with a significantly high clinical burden with major ramifications on the quality of life. Several complications associated with DM can lead to severe loss of visual acuity or even blindness. DM is also associated with several structural and functional alterations of the human cornea, such as corneal epithelial changes, morphological changes to the corneal nerves, decreased endothelial cell density, reduced corneal sensitivity and altered central corneal thickness (Schultz et al., 1981; Schultz et al., 1983; Herse, 1988; Rosenberg et al., 2000; Bardsley and Want, 2004; Rother, 2007; Skarbez et al., 2010; Priyadarsini et al., 2016a,b).

The cornea, the outermost layer of the eye responsible for focusing our vision, is a clear, highly organized tissue devoid of any blood vessels. (Busted et al., 1981; Rosenberg et al., 2000; Holmes et al., 2001; Robert et al., 2001; Gekka et al., 2004; Eghrari et al., 2015; Meek and Knupp, 2015). The cornea has five layers consisting of the epithelium, Bowman's membrane, stroma, Descemet's membrane, and the endothelium. The thickest layer of the cornea is the stroma which primarily consists of collagen and the resident cells termed keratocytes (Busted et al., 1981; Robert et al., 2001; Gekka et al., 2004; Eghrari et al., 2015; Meek and Knupp, 2015). The architecture of the stroma layer is responsible for the majority of the corneal transparency, structure and integrity.

The cornea is the most densely innervated tissue of the human body (Shaheen et al., 2014; Eghrari et al., 2015) and several studies agree that corneal nerve dysfunction is one of the common pathobiological features that's commonly seen with DM (Shaheen et al., 2014). The healthy and diseased state of the cornea is closely related to neuronal innervation, as the distribution of innervation is critical in maintaining corneal transparency and structure (Marfurt et al., 2010; Bikbova et al., 2016; Wang et al., 2017). Diabetic changes in corneal nerves have primarily been associated with morphological alterations of the subbasal nerve plexus in both retinopathy and peripheral neuropathy (Tavakoli et al., 2011; Nitoda et al., 2012).

There have been various animal models for both T1DM (Nerup et al., 1994; Lenzen et al., 2001; Mathews et al., 2002; Yang and Santamaria, 2006; Zhou et al., 2011) and T2DM (Hummel et al., 1966; Surwit et al., 1988; Ikeda, 1994; Phillips et al., 1996; Leiter and Reifsnnyder, 2004; Clee and Attie, 2007; Yoshida et al., 2010; Gault et al., 2011; Park et al., 2011) as reviewed by King (King, 2012). Although animal models that mimic diabetic corneal changes provide a good representation of DM, they have had limitations when translating these findings to human treatments (Schwartz, 1974; Hyndiuk et al., 1977; Schultz et al., 1981; Rosenberg et al., 2000; Dogru et al., 2001; Yoon et al., 2004; Xu et al., 2009). Despite the large number of studies reported to-date, the interactions and specific mechanisms involved in the complications associated with DM including diabetic keratopathy and diabetic neuropathy are not well understood. Furthermore, there have been

very little studies involving the interactions between the corneal stroma and the stromal nerves. One of the reasons for this is the lack of human *in vitro* models that can successfully mimic what is seen *in vivo*. One of the most advanced *in vitro* models perhaps is the organotypic cultures, developed by Ljubimov and co-authors (Kabosova et al., 2003; Winkler et al., 2014) that mainly focuses on the identification of epithelial defects. Given that both cell types are damaged in DM, our model includes a 3D co-culture model of human corneal stromal cells and neurons which allows us for the first time to investigate the interactions between the two cell types within the human tissue.

In this study we developed a novel 3D *in vitro* model that mimics the *in vivo* stromal-nerve interactions during healthy and DM conditions. We co-cultured healthy human fibroblast cells, T1DM cells, and T2DM cells on along with the SH-SY5Y cell line that was further differentiated into human neuronal cells on our 3D system. The SH-SY5Y cell line was used for this study, as it is one of the most established, well characterized lines used to study neuronal growth. This model is the first of its kind and may provide new insights for a better understanding of the corneal complications associated with DM. Furthermore, this model could assist in the development of novel therapeutic agents for the treatment of diabetic keratopathy and diabetic neuropathy complications.

2. Materials and methods

2.1. Ethics and inclusion criteria

All procedures adhered to the tenets of the Declaration of Helsinki. Healthy human corneal samples were obtained from the National Disease Research Interchange (NDRI) and the diabetic corneal samples were obtained from Oklahoma Lions Eye Bank. The Oklahoma University Health Sciences Centre Institutional Review Board (IRB) was notified and appropriate permission was obtained prior to experimental initiation. Inclusion criteria for the diabetic donors included clinical diagnosis of T1DM or T2DM and absence of other unrelated diseases or ocular pathology. The healthy/control group included corneal samples from donors with no history of ocular trauma or systemic diseases. For each group in this study, cells were isolated from at least 4 donor corneal tissue samples (n = 4). Diabetic related complications like acute cerebral infarction, cerebrovascular accident, complications from end stage renal disease, or respiratory failure were the causes of death for the diabetic groups whereas the cause of death for healthy controls varied from accidental to non-diabetic related diseases like head trauma, acute segment elevation myocardial infarction, subarachnoid hemorrhage and cardiac arrest. In this study the average age for each group was as follows: Healthy: 57y/o, T1DM: 55y/o, and T2DM: 59y/o with no significant age differences noted between the groups. The duration of DM was from 3 to 30 years, with a mean of 15.5 years.

2.2. Primary cell isolation and culture

HCFs, T1DMs and T2DMs cells were isolated as per our previously optimized protocol. Initially the corneal epithelium and endothelium are removed from the stroma by scraping with a razor blade. Further, the stromal tissues are cut into small pieces of size $\sim 2 \times 2$ mm and placed into T25 culture flasks. This is followed by making the explants adhere to the

bottom of the flask at 37 °C for about 30 min where Eagle's Minimum Essential Medium (EMEM: ATCC: Manassas, VA) containing 10% fetal bovine serum (FBS: Atlantic Biological's; Lawrenceville, CA) and 1% Antibiotic (Gibco Antibiotic-Antimycotic, Life technologies) is added to the flasks carefully without disturbing the explants. Upon 100% confluence the cells are passaged into T75 culture flasks and further cultured before being processed for further analysis (Karamichos et al., 2012; Priyadarsini et al., 2014).

2.3. 3D construct assembly, maintenance, and co-cultures

Our 3D constructs for HCFs, T1DM, and T2DM cells were assembled as per our previously optimized protocol (Karamichos et al., 2009; Karamichos et al., 2010; Karamichos et al., 2011a,b; Karamichos et al., 2012). 1×10^6 cells/well were seeded on poly-carbonate membrane inserts with 0.4- μ m pores (Corning Costar; Corning Incorporated, Corning, NY, USA) and cultured in EMEM containing 10% FBS and 1% antibiotic (Fig. 1). Further, the cells were treated with 0.5 μ M 2-O- α -D-glucopyranosyl-L-ascorbic acid (American Custom Chemicals Corporation, San Diego, CA, USA) to stimulate ECM secretion and assembly. The cultures were maintained for 3 weeks and fresh media was supplemented every other day during the entire study period.

After 3 weeks, 8×10^3 cells/well of SH-SY5Y human neuro-blastoma cells were seeded on top of our assembled 3D constructs and differentiation was initiated (Fig. 1). SH-SY5Ys were treated with 10 mM Retinoic Acid (Sigma Aldrich, USA) which was added to EMEM containing 1% FBS and cultured for 5 days. Cells were then switched to a treatment of serum free media of EMEM containing 2 nM of Brain-derived neurotrophic factor (BDNF) (Sigma Aldrich) and cultured for 2 additional days before they were processed for qRT-PCR and Immunofluorescence.

2.4. Real-time PCR

All samples were evaluated for mRNA expression by qRT-PCR as previously described (Karamichos et al., 2011a,b; Karamichos et al., 2014a,b; Priyadarsini et al., 2014). Briefly, Ambion RNA mini extraction kit (Ambion TRIzol Plus RNA Purification Kit: Life technologies, Carlsbad, CA) was used to extract total RNA. This was followed by synthesis of cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. We used TaqMan gene expression of both (Applied Biosystems, Foster City) GAPDH (Hs99999905_m1) and 18S (Hs99999901_s1) as our housekeeping genes in the HCFs, T1DM, and T2DM. Furthermore, no changes were noted in either one between the three cell types in our housekeeping genes. ACTA2 (Hs00426835_m1), COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), COL5A1 (Hs00609133_m1), IGF1 (Hs01547654_m1) and IGF1R (Hs00609566_m1), NRF1(Hs00192316_m1), PGC1A (Hs01016719_m1) and TFAM (Hs00273372_s1) were investigated.

2.5. Embedding and staining of semithin sections

All constructs were fixed with 4% Paraformaldehyde and 2% Glutaraldehyde in 0.1 M Sodium Cacodylate buffer and processed for embedding. Further, the samples were post fixed in 1% Osmium tetroxide in Sodium Cacodylate, and then rinsed with 0.1 M Sodium

Cacodylate buffer. Cells were then held in 0.1 M Sodium Cacodylate buffer for 3 days and thereafter the samples were dehydrated in a graded acetone series as follows: 50%, 60%, 75%, 85%, 95%, 100%. Next, the cell constructs were treated with 100% Propylene Oxide. Following dehydration, the samples were infiltrated in a graded Epon/Araldite (EMS) resin/Propylene Oxide series (1:3, 1:1, 3:1) for 60 min, 120 min, and overnight respectfully. Samples were further infiltrated the following day with pure resin for 45 min, 90 min, and then overnight. The cell constructs were then embedded in resin plus BDMA (accelerator) and polymerized at 60 °C for 48 h as per the previously reported methods (Zieske et al., 1994; Karamichos et al., 2010; Karamichos et al., 2011a,b). Ultra-thin sections of 500 µm thickness were made and further stained with toluidine blue and rinsed 3X with water before viewing on a Hitachi H7600 TEM at 80 kV equipped with a 2 k × 2 k AMT digital camera.

2.6. Immunofluorescence

All constructs were initially fixed with 4% paraformaldehyde in PBS (Guo et al., 2007; Ren et al., 2008; Karamichos et al., 2010; Karamichos et al., 2011a,b) and further processed for cytochemical analysis. Furthermore, sample permeabilization was initiated by using PBS containing 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and blocking with 1% BSA (Thermo Scientific) in PBST (0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) PBS) containing 0.3 M glycine (Fischer Scientific). Samples were incubated overnight at 4 °C with Anti-Nestin (Abcam, USA) primary antibody and stained with DAPI (1 µg/ml, Life Technologies, USA). The samples were later imaged using a confocal microscope (Olympus Confocal FV 500, USA).

2.7. Statistical analysis

Statistical data analysis was conducted using Graph Pad Prism 6. One-way ANOVA was used for the entire study and $p < 0.05$ was considered statistically significant. The data generated was from three independent experiments with each set repeated at least 4 times.

3. Results

3.1. Differentiation of SH-SY5Y neuroblastoma cells into neuronal cells

Various studies have already shown successful usage of SH-SY5Y neuroblastoma cells and their ability to differentiate into functionally mature neurons (Encinas et al., 2000; Agholme et al., 2010; Schneider et al., 2011). In this study, our first priority was to generate viable neuronal cells with morphological characteristics seen in mature human neurons. In order to do so, 2D cultures were initially established. SH-SY5Ys were differentiated in the presence of RA and BDNF. Results indicated neuronal morphology with extensively long, branched neurons as observed by phase contrast microscopy using the neuronal marker Nestin (Fig. 1B and D). These cells were clearly different from the undifferentiated controls (Fig. 1A and C) that did not possess any of the neuronal morphological characteristics.

3.2. 3D co-culture assembly

In this study our aim was to establish a novel 3D *in vitro* model that would mimic the *in vivo* stromal-nerve interactions in DM. Fig. 2 shows successful development of HCF-SH-SY5Y co-cultures. Representative semithin section images are shown for HCF Controls (Fig. 2A)

and HCF co-cultures (HCF-C) (Fig. 2B), demonstrating the feasibility of this principle. Fig. 2C shows neuronal cells growing on top of HCFs with extended neuron extensions mirroring the morphology usually seen *in vivo*. Due to the lack of investigation of corneal stromal nerve interactions, our model shows promise in the potential use of mapping out the underlying mechanisms associated with these corneal stromal nerves in DM.

3.3. Analysis of neural markers

We further evaluated the expression of some of the essential neural markers through RT-PCR including Nestin, a neural progenitor marker, β III Tubulin, an early neuronal marker, and Substance P, a neuropeptide belonging to the tachykinin family with very specific neural activities. All co-culture constructs were compared to constructs without the neuronal layer in order to determine the effect the neural cells had on the expression of these markers in both cell types.

Our results showed upregulated β III Tubulin (Fig. 3A) expression in the co-cultures of all three cell types when compared to their respective controls. Significantly upregulated β III Tubulin expression was observed only in T2DM co-culture (T2DM-C) when compared to HCF control and HCF-C ($p = 0.01$) and also T1DM control and T1DM co-culture (T1DM-C) ($p = 0.01$) (Fig. 3A). Nestin expression was upregulated in all co-cultures for all the three cell types, but did not reach significance. In case of Substance P, T1DM-C showed significant upregulated expression when compared to most conditions (Fig. 3C; $p = 0.05$). Interestingly, T2DM-C showed significant downregulation in comparison to the T1DM-C (Fig. 3C; $p = 0.05$) indicating that there are potential physiological differences between stromal and neuronal interactions in T1DM and T2DM.

The significant modulation of both β III Tubulin and Nestin, in the co-cultures, suggests active interactions between the stromal and neuronal cells. Furthermore, it highlights significant differences between the crosstalk of stromal and neuronal cells in a Healthy versus a T1DM/T2DM stroma.

3.4. Specific fibrotic markers expression

The corneal stroma is primarily composed of collagens, crystallins and proteoglycans. In general, the ECM is made of exquisitely aligned and organized collagen, particularly Collagen-I and -V being the two main collagens present in a normal healthy cornea (Karamichos et al., 2010). Collagen-III and α -SMA expression are often upregulated during injury or infection, indicating corneal fibrosis and scarring (Karamichos et al., 2010). In this study we quantified the expression of Collagen-III and α -SMA (Fig. 4) in both controls as well as the respective co-cultures.

Interestingly, we found a significant upregulation in Collagen-III in T2DM control when compared to almost all conditions (Fig. 4A; $p = 0.05$) except for T2DM-C. α -SMA, a marker for myofibroblasts, did not reach significance in any of the conditions. However, the bigger difference was seen between T2DM control and T2DM-C (Fig. 4B; $p < 0.07$).

3.5. Insulin growth factor and its receptor activity

Insulin growth factor 1 (IGF1) and its receptor (IGF1-R) are key modulators during various vital cellular processes including cellular differentiation, proliferation, survival and metabolism in various array of cell types (Ljubimov, 2017). They are also known to play a role in DM as well as diabetic keratopathy (Ljubimov, 2017). In this study we found significant upregulation of IGF1 expression in T2DM control (Fig. 5A; $p = 0.001$) when compared to all other conditions except for T2DM-C where a significant downregulation was observed (Fig. 5A; $p = 0.01$) indicating that there may be some differential modulation of IGF1 based on the presence of the neurons.

Surprisingly, IGF1-R expression had an entirely opposite expression. We found a significant upregulation in T2DM-C when compared to T2DM control (3.5 fold, Fig. 5B; $p = 0.0001$). IGF1-R was also significantly upregulated in T2DM-C compared to HCF-C (3.5 fold, $p = 0.0001$) as well as T1DM-C (1.5, $p = 0.01$). Slightly elevated expression of IGF1-R was also witnessed in both HCF-C and T1DM-C when compared to their respective controls but no significance was reached.

The exact role of IGF-1 and IGF1-R within the human cornea and during diabetic keratopathy remains elusive. Our data can be vital in giving us a better understanding about the corneal state in healthy and DM-like conditions.

3.6. Mitochondrial gene expression

In our previous study we have already reported severe structural mitochondrial dysfunction in T1DM and T2DM constructs when compared to healthy controls (Priyadarsini et al., 2016a,b). In this study our goal was to further delineate the mitochondrial damage at the basal cellular level and assess the length at which the mitochondria gets affected when neurons are present. We analyzed the gene expression of several mitochondrial genes including NRF1, PGC-1 α and TFAM.

Our data revealed an increased expression of NRF1 in all co-culture groups for the three cell types but no significance was observed (Fig. 6A). However, significant upregulation of PGC-1 α expression was observed in both T1DM control ($p = 0.001$) and T2DM control ($p = 0.01$) when compared to the HCF control. Interestingly, all the co-culture groups showed downregulated expression of PGC-1 α when compared to their respective controls (Fig. 6B). On the contrary, only T1DM control showed significance when compared to T1DM-C (Fig. 7B; $p = 0.01$). TFAM expression was highest in the HCF-C with significant upregulation when compared to its respective HCF control (Fig. 6C; $p = 0.01$) and downregulation when compared to all DM constructs.

Based on these mitochondrial markers, there seems to be a difference between healthy and DM constructs as well as the controls when comparing them to their respective co-culture constructs. These results indicate that different compartments of the DM cell mitochondria are affected and further studies are necessary to investigate these underlying mechanisms.

4. Discussion

Ocular complications associated with DM have a tremendous impact on the visual acuity of a large population throughout the world. These complications include numerous clinical outcomes such as corneal thickness (Busted et al., 1981; Lee et al., 2006a,b; Su et al., 2008), corneal epithelial lesions (Schultz et al., 1981; Schultz et al., 1983), delayed wound healing and repair mechanisms (Mishima, 1957; Alper, 1975; Baker et al., 1993; Araki et al., 1994), reduced corneal sensitivity, corneal nerve damage and endothelial dysfunction (Saini and Mittal, 1996), as well as weakening of the epithelial barrier (Saini and Khandalavla, 1995) which often leads to corneal infections and stromal fibrosis (Gobbels et al., 1989; Gekka et al., 2004). Despite all of the defects associated with DM such as fibrosis, erosions, and thinning (Karamichos et al., 2010), the corneal stroma in DM is severely under investigated. There are numerous studies that have successfully developed various animal models (Lenzen et al., 2001; Mathews et al., 2002; Leiter and Reifsnnyder, 2004; Yang and Santamaria, 2006; Clee and Attie, 2007; Yoshida et al., 2010; Gault et al., 2011; Park et al., 2011) as well as a small number of *in vitro* studies focusing on corneal nerve pathologies and corneal sensitivity (Schwartz, 1974; Hyndiuk et al., 1977; Schultz et al., 1981; Rosenberg et al., 2000; Dogru et al., 2001; Yoon et al., 2004; Xu et al., 2009). While these studies have significantly increased our understanding of the pathophysiology of DM in the cornea, it would be of great benefit to have an *in vitro* human model to closely represent what is seen *in vivo*. The cornea represents tremendous benefits towards the investigation of diabetic pathology due to its essential advantages of clarity, ease of observation, accessibility, and lack of cellular complexity.

In this study, we aimed to develop an *in vitro* corneal stromal-nerve model that would mimic what is seen *in vivo* in terms of cellular mechanisms to provide a tool for a better understanding of changes associated with DM pathology in the stroma. DM is a very complex disease, and there is very little known about the crosstalk between the corneal stroma and stromal nerves. Most studies concerning the diabetic changes of corneal nerves to this point primarily relate to the subbasal nerves, with much less research focusing on the stromal nerves. While pharmacological treatment for diabetic keratopathy is still not available, the current *in vitro* model has the potential to provide solid foundations towards the discovery of new treatments. Preservative-free artificial tears, topical antibiotics, bandage contact lenses, amniotic membrane transplantation, tarsorrhaphy, and a conjunctival flap are useful methods but they are not a cure (Sacchetti and Lambiase, 2014). Therefore, the establishment of a treatment is of utmost importance.

It is well recognized that impaired corneal innervation in DM is a key indicator early in the pathogenesis of diabetic keratopathy. Diabetic keratopathy has long been proposed as a sign of peripheral neuropathy, and it has been justified by later studies relating an association between corneal epithelial changes and manifestations of diabetic neuropathy as reviewed by Ljubimov. Furthermore, Misra et al. described clinically using *in vivo* confocal microscopy in patients with T1DM (Misra et al., 2012) corneal nerve parameters confirming a decrease in the sub-basal nerve density and corneal sensitivity in diabetic patients. They also found a significant relationship between corneal neuropathy and systemic neuropathy. According to a previous study, a decreased epithelial nerve density may result from the abnormalities of

stromal nerve architecture affected by five or more years of insulin-dependent DM (He and Bazan, 2012). Due to the extensive damage associated with the epithelial layer in diabetic keratopathy, the corneal stromal association must be more extensively studied to gain a better understanding of key underlying mechanisms.

Our approach showed significant promise with differentiated neuronal cells on top of corneal stromal self-assembled ECM with elongated neurons and expression of neuronal markers.

In our previous studies we have already reported the significance of collagen secretion and assembly using our 3D model (Karamichos et al., 2007a,b; Karamichos et al., 2012; Karamichos et al., 2014a,b; Karamichos, 2015; Priyadarsini et al., 2016a,b). Collagen I and V are the dominant types present within a healthy cornea, whereas Collagen III is associated with fibrosis (Karamichos et al., 2010); it results mainly due to the transformation of keratocytes to myofibroblasts in response to injury or disease. Patients with diabetic keratopathy often exhibit corneal scarring or ulcers, which is primarily due to the prolonged exposure to a hyperglycemic environment, as well as a large amount of reactive oxygen species leading to keratocyte activation. In our previous study, we have shown that the human diabetic corneal stroma has elevated levels of Collagen-III, supporting the clinical manifestation of increased scarring in patients with diabetic keratopathy (Brightbill et al., 1978). Interestingly, in this study, we observed a significant increase in Collagen-III in T2DM controls and a downregulation of T2DM-C, suggesting that corneal fibrosis is linked to corneal stromal interactions with the nerves. A similar pattern was seen with α -SMA expression, but it did not reach significance ($p < 0.07$).

Furthermore, IGF1 and its receptor IGF1-R are well known for their role during several key cellular processes in the human cornea. IGF-1 is classified as a multifunctional regulatory peptide that shares structural homology with proinsulin. It mediates multiple functions in the body including proliferation, differentiation, and survival effects, depending on the target cell and the presence of other hormones and growth factors (Gockerman et al., 1995). One study demonstrated that IGF-1 induces human corneal epithelial cell migration through upregulation of L α -5 and β 1 integrin protein expression via the PI3-K/AKT pathway as well as cross-talk between IGF-1 associated PI3 kinase and ECM proteins in several cell types (Lee et al., 2006a,b). IGF1 has well known effects within the cornea on proteoglycan biology in addition to modulating keratocytes and corneal stromal extracellular matrix synthesis (Hassell et al., 2008; Berthaut et al., 2011). Despite many recent studies on IGF1 and its receptor in the cornea, the role these molecules play in the interactions between the stroma and nerves is severely under investigated. Our study revealed a significant increase in IGF1 expression in T2DM controls when compared to our HCF controls, while the presence of the nerves (T2DM-C) appeared to interplay with the T2DM cells and dropped the IGF1 expression down towards the HCF control levels. The opposite effect was observed in IGF1-R expression where T2DM-C was higher when compared to T2DM controls. Studies have shown that the effects of IGF1 on the regulation of gene expression is primarily achieved through IGF1-R activation and activation of IGF1-R often leads to regulation of gene expression, protein synthesis, cell proliferation/death and glucose and lipid metabolism (Boucher et al., 2010). Our data clearly shows interplay between the stromal ECM and the neurons in an IGF1/IGF1-R dependent mediated pathway.

Previously we have already reported severe structural mitochondrial dysfunction (Priyadarsini et al., 2016a,b) and in this study we analyzed the expression of a few important mitochondrial biogenesis factors like NRF1, PGC-1 α and TFAM. PGC-1 α has been established as the master regulator of mitochondrial biogenesis through its relationship with NRF1 and NRF2 and also by increasing NRF1 and NRF2 expression levels and activities through protein-protein interactions (Li et al., 2017). NRF1 and NRF2 activate another important mitochondrial factor known as TFAM and also bind to promoter regions of nuclear genes encoding subunits of the five complexes in the mitochondrial electron transport chain (Uittenbogaard and Chiaramello, 2014). The activation of this pathway involving PGC-1 α , NRF, and TFAM leads to substantial changes within the mitochondrion including the synthesis of mitochondrial DNA and proteins as well as the eventual generation of new mitochondria (Li et al., 2017). PGC-1 α is (Scarpulla 2011) often activated by Reactive oxygen species (ROS), Reactive Nitrogen species (RNS), and cAMP response element-binding (CREB) proteins (Fernandez-Marcos and Auwerx, 2011). In this study we observed significant increase in its expression in T1DM and T2DM controls when compared to the HCF controls as well as their respective co-cultures. This data suggests significant interactions when the neurons are present including the potential recovery of mitochondrial function thus minimizing damaging effects of DM. TFAM, an important mitochondrial transcription factor that functions during mitochondrial genome replication, has various clinical significances in various diseased conditions like pulmonary disease or cancer. Our study showed significant increase in HCF co-culture when compared to its control and to that of the diabetic cells and its co-culture. Thus, aberrant mitochondrial biogenesis can certainly decide the cell proliferation rate and effective functioning of cells.

A few potential limitations of our study relate to the use of neuroblastoma cells to mimic corneal neural cells and the use of “healthy” neurons to mimic the disease state in DM. However, it is difficult to obtain and culture DM neurons at this time, and this is the first and only 3D *in vitro* model to investigate the neuronal interactions and signaling with the diabetic corneal stroma. Overall, our 3D *in vitro* model may provide a number of conceptual innovations for the development of future therapeutic discoveries as well as provide a better understanding of the underlying mechanisms associated with corneal stromal nerve interactions related to DM complications.

5. Conclusions

DM is a significant clinical burden with devastating effects on humans including many complications associated with vision. The model discussed here is surprisingly simple, but it has the potential to be highly innovative. One of the significant advantages is that the corneal stromal cells are allowed to secrete and assemble their own ECM instead of being seeded onto an artificial environment. Based on the data shown, as well as previous studies from our group, the ECM assembled from HCFs is different from the one assembled from T1DMs and T2DMs. This suggests that corneal stromal cells are able to maintain all or at least part of their *in vivo* dysfunction while cultured *in vitro*. This is rather intriguing if we consider that the *in vitro* environment provides all the nutrients and supplements a cell needs in order to be healthy. The use of neuroblastoma cells presents as a potential limitation to the study, but they are a good starting point for providing us a better understanding of the crosstalk

between corneal stromal and neural cells. Due to the lack of studies concerning the interactions between these corneal stromal cells and nerves, this model has the potential to contribute considerable insight regarding the underlying mechanisms associated with damage to the diabetic corneal stroma. Further studies must be conducted to determine if results are consistent across all DM patients or gene regulation varies based on gender and age. In future studies, these *in vitro* self-assembled tissues may serve as drug testing platforms for current or new therapeutic agents.

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Fig. 1.

A) Phase contrast microscopy image of SHY-SY5Y neuroblastoma cells before differentiation B) Immunofluorescence image of SHY-SY5Y neuroblastoma cells before differentiation stained for Nestin where the marked area shows no extended neurons C) Phase contrast microscopy image of differentiated neuronal cells forming neuronal network with neuron extension. D) Immunofluorescence image of differentiated neuronal cells where marked area showing extended neuron extension which is a visual indicator of successful cell differentiation and also stained positive for Nestin.

Fig. 2. Representative stained semithin section images for A) Human corneal fibroblast B) Human corneal fibroblast + Human corneal neuronal cell co-cultures C) Neuronal cells seeded on top of corneal stromal fibroblast cells stained for Nestin and DAPI. The thickness of each sections were 500 μm.

Fig. 3. mRNA expression of nerve cell markers A) β III Tubulin B) Nestin and C) Substance P. (A–C) Gene expression quantification, normalized to the loading control. For HCFs, T1DMs and T2DM controls and co-cultures n = 4. One-way ANOVA was performed (* = p < 0.05; ** = p < 0.01; *** = p < 0.001) and error bars represent standard error of the mean.

Fig. 4.

Fibrotic markers expression for HCF, T1DM and T2DM controls and their co-cultures are shown: A) Col III and B) α -SMA. (A–B) Gene expression quantification, normalized to the loading control. For HCFs, T1DMs and T2DM controls and their co-cultures $n = 4$. One-way ANOVA was performed ($* = p < 0.05$) and error bars represent standard error of the mean.

Fig. 5. mRNA expression of A) IGF1 and B) IGF1-R (A–B) Gene expression quantification, normalized to the loading control. For HCFs, T1DMs and T2DM controls and their co-cultures $n = 4$. One-way ANOVA was performed (** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$) and error bars represent standard error of the mean.

Fig. 6. mRNA expression of mitochondrial probes A) NRF1 B) PGC-1 α and C) TFAM. (A–C) Gene expression quantification, normalized to the loading control. For HCFs, T1DMs and T2DM controls and their co-cultures n = 4. One-way ANOVA was performed (** = p 0.01; *** = p 0.001) and error bars represent standard error of the mean.

Fig. 7. mRNA expression of mitochondrial probes A) NRF1 B) PGC-1 α and C) TFAM. (A–C) Gene expression quantification, normalized to the loading control. For HCFs, T1DMs and T2DMs n = 4. One-way ANOVA was performed (** = p < 0.01; *** = p < 0.001) and error bars represent standard error of the mean.