Differential expression of epithelial basement membrane components nidogens and perlecan in corneal stromal cells in vitro

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Purpose: The purpose of this study was to examine the expression of corneal epithelial basement membrane (EBM) components in different corneal stromal cell types. In vitro model systems were used to explore the expression of EBM components nidogen-1, nidogen-2, and perlecan that are the primary components in the lamina lucida and the lamina densa that defectively regenerate in corneas with stromal opacity after in −9.0 D photorefractive keratectomy (PRK).
Methods: Primary rabbit corneal stromal cells were cultured using varying serum concentrations and exogenous growth factors, including fibroblast growth factor (FGF)-2 and transforming growth factor (TGF)-β1, to optimize the growth of each cell type of interest. The expression of the keratocyte-specific marker keratocan and the myofibroblast-specific marker α-smooth muscle actin (α-SMA) were analyzed with real-time PCR, western blot, and immunocytochemical staining to evaluate the specificity of the cell types and select optimal conditions (high keratocan and low α-SMA for keratocytes; low keratocan and high α-SMA for myofibroblasts; low keratocan and low α-SMA for corneal fibroblasts). The expression of the EBM components nidogen-1, nidogen-2, and perlecan was evaluated in each corneal cell type using real-time PCR, immunostaining, and western blotting. In agreement with previous studies, serum-free DMEM was found to be optimal for keratocytes, DMEM with 10% serum and 40 ng/ml FGF-2 yielded the best marker profile for corneal fibroblasts, and DMEM with 1% serum and 2 ng/ml TGF-β1 was found to be optimal for myofibroblasts.
Results: Nidogen-1 and nidogen-2 mRNAs were highly expressed in keratocytes, whereas perlecan was highly expressed in myofibroblasts. In keratocytes, nidogen-2 and perlecan proteins were expressed predominantly in intracellular compartments, whereas in myofibroblasts expression of both EBM components was observed diffusely throughout the cell. Although the perlecan mRNA levels were high in the myofibroblasts, the qualitative protein expression was different from that of the keratocytes. Corneal fibroblasts produced a low amount of each EBM component.
Conclusions: We have demonstrated qualitative and quantitative differences in the expression of nidogen-1, nidogen-2, and perlecan by keratocytes compared to myofibroblasts that may contribute to defective regeneration of the lamina lucida and the lamina densa of the EBM associated with late stromal haze after high-correction PRK.

Recent studies have linked defective epithelial basement membrane (EBM) lamina lucida and lamina densa regeneration in the cornea to the development of late stromal haze after photorefractive keratectomy [1,2]. EBMs have critical roles in modulating growth factor–mediated stromal–epithelial interactions in organs throughout the body, including the cornea. The basement membrane (BM) is a 50 nm to 100 nm thick layer of specialized extracellular matrix (ECM) protein complex found basolateral to all epithelium and endothelium [3]. The BM composition is extremely diverse, tissue-specific, and dynamic, and the composition and assembly of the BM in a tissue vary according to the tissue's physiologic and/or pathophysiologic state [4-6].

The corneal EBM is positioned between basal epithelial cells and the stroma [2]. Many studies have indicated that the corneal EBM is more than a thin acellular layer separating epithelial cells from the adjacent anterior stroma. Since the corneal stroma is avascular and has a low keratocyte density, it is likely that the corneal EBM is different in composition from the BM in other tissues [2,5,7]. The corneal epithelial BM has regional heterogeneity from the central cornea to the limbus to the conjunctiva [2,8]. The corneal EBM is assembled from four primary components: collagens, laminins, heparan sulfate proteoglycans (HSPGs), and nidogens, although many other components such as fibronectin are also present, which may be tissue specific [5,7]. The lamina lucida and lamina densa layers that are not regenerated in rabbit corneas with haze [1] are composed of specific components that include perlecan, nidogen-1 and -2, and laminin 332 [6]. Perlecan (HSPG2) is a basement membrane–specific HSPG protein that has been identified in the EBM [9,10] that has important functions in maintaining cell adhesion and the integrity of the corneal matrix [11]. Perlecan deficiency impairs the corneal epithelial structure in a mutant mouse

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model [12]. Nidogens interact with laminins [13,14], collagen IV, and perlecan, and thus act as bridging molecules in the EBM [15,16].

Evidence has been provided for a stromal cellular origin for some EBM components in the cornea [17-20]. Changes in the qualitative or quantitative composition, localization, or structure of EBM proteins have been shown to be involved in the pathophysiology of different disease states [7]. Corneal stromal keratocytes are relatively quiescent in the fully-developed normal cornea. However, after injury or infection to the cornea, growth factors, and cytokines originating from corneal epithelial cells, inflammatory cells, and tear fluid activate the keratocytes to fibroblastic cells and, in some situations where severe opacity or scarring is noted, to myofibroblast phenotypes [21-25]. These cells synthesize disorganized extracellular matrix that contributes to pathological stromal opacity. Each cell type has been characterized by the specific marker proteins the cell expresses [26-29]. Keratocan is limited to the corneal stroma, and keratocan expression is considered a phenotypic marker for keratocytes [26,27,30-34]. Lumican is the major keratan sulfate proteoglycan of the cornea but is also distributed in interstitial collagenous matrices throughout the body. Lumican regulates collagen fibril organization, circumferential growth, corneal transparency, epithelial cell migration, and tissue repair, and is used as a marker for keratocytes [35,36]. Alpha-smooth muscle actin (α-SMA) is the primary marker of myofibroblasts [32,37].

EBM composition and formation are altered during corneal wound healing after injury, infection, and surgery associated with the generation of fibroblasts and myofibroblasts. It is important to understand the contributions each corneal stromal cell type (keratocyte, fibroblast, and myofibroblast) may make to the regeneration of the EBM. The current study focused on identifying the expression patterns of nidogens and perlecan components found in the lamina lucida and the lamina densa of the EBM in the three dominant keratocyte-derived corneal stromal cell types in vitro since

these are the primary components in the lamina lucida and the lamina densa of the EBM [1,38].

METHODS

Primary cell culture: Fresh rabbit corneas were obtained from Pel Freeze (Rogers, AR). Twenty-five rabbit corneas were used in each experiment to have sufficient stromal cells for the experiments. Each experiment was repeated three times with a new set of primary culture cells from rabbit corneas. The epithelial and endothelial layers were removed from the corneas using 0.12 mm forceps and a #64 scalpel blade (BD Beaver, Franklin Lakes, NJ) under a dissecting microscope using the sterile technique. Keratocytes were isolated from the corneal stroma using a modified Jester et al. procedure [14]. Briefly, the corneas were digested in sterile Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 2.0 mg/ml collagenase (Gibco, Grand Island, NY) and 0.5 mg/ml hyaluronidase (Worthington, Lakewood, NJ) overnight at 37 °C. Cells were spun down and cultured in DMEM (Gibco), supplemented with RPMI vitamin mix (Sigma), non-essential amino acids (Gibco), antibiotic antimycotic solution (Gibco), sodium bicarbonate (JRH Bioscience, Lenexa, KS), and L-ascorbic acid (Sigma, St. Louis, MO). Keratocytes were grown with different serum concentrations—1%, 5%, or 10% fetal bovine serum (FBS; Invitrogen Corporation, Carlsbad, CA), with or without fibroblast growth factor (FGF-2, 40 ng/ml, Sigma Aldrich, St. Louis, MO), heparan sulfate (HS; 5 µg/ml), and TGF-β1 (2 ng/ml, R&D, Minneapolis, MN) for 60–72 h. The culture medium was changed every 48 h. Corneal cells between one and three passages were used for all experiments. The conditions used for the cell culture are summarized in Table 1 and confirmed in Appendix 1 and Appendix 2.

Immunocytochemistry: For immunocytochemical analyses, the cells were fixed with 4% paraformaldehyde (Sigma) for 30 min and permeabilized with 0.2% Triton X-100 (Sigma) in PBS (7.6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 160 mM NaCl, pH

TABLE 1. LIST OF CULTURE CONDITIONS USED.		
Culture Condition	**Serum**	**FGF2/HS/TGF-β**
1	Serum Free (SF)	Nil
2	Serum Free (SF)	FGF2–40ng/ml; HS-5µg/ml
3	1% FBS	FGF2–40ng/ml; HS-5µg/ml
4	5% FBS	FGF2–40ng/ml; HS-5µg/ml
5	10% FBS	FGF2–40ng/ml; HS-5µg/ml
6	1% FBS	TGF-β −2ng/ml

FBS=Fetal Bovine Serum, FGF2=Fibroblast Growth Factor2, HS=Heparan Sulfate TGF-β=Transforming Growth Factor beta.

7.4) for 10 min, followed by blocking with 5% bovine serum albumin (BSA; Sigma) or 5% FBS (Gibco) in PBS for 1 h. The cells were then treated with primary antibody for 90 min at room temperature and washed three times in PBS, followed by treatment with secondary antibody for 1 h.

For nidogen-2 and perlecan, the slides were treated with primary antibody for overnight at 4 °C and washed three times in PBS, followed by treatment with a secondary antibody for 1 h at room temperature. Slides were rinsed with PBS, and coverslips were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA). The primary antibodies included a mouse monoclonal anti-human α-smooth muscle actin clone 1A4 (1:100, M0851, Dako, Carpinteria, CA), mouse monoclonal anti-bovine perlecan A71 antibody (1:150, CSI 001–71–02, Thermo Scientific, Rockford, IL), and goat anti-human nidogen-2 antibody (1:100, AF3385, R&D Systems, Minneapolis, MN). Antibodies that recognized rabbit nidogen-1 could not be identified; therefore, the isoform of the nidogen protein could not be studied. The secondary antibodies used in this study were goat anti-mouse immunoglobulin G (IgG) Alexa Fluor 568 (1:200, A11004, Life Technologies, Carlsbad, CA) and donkey anti-goat IgG fluorescein isothiocyanate (FITC; 1:100, SC-2024, Santa Cruz). Fluorescence images were captured using a Leica DM5000 microscope (Buffalo Grove, IL) equipped with Q-imaging Retiga 4000RV (Surrey, Canada) camera and ImagePro software (Leica).

Extraction of total RNA, synthesis of cDNA, and RT–PCR: Total RNA was isolated from corneal cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized from total RNA (approximately 400 ng) with the ThermoScript RT–PCR system (Invitrogen, Carlsbad, CA) using oligo (dT) primers according to the manufacturer's instructions. Initial screening of cDNA for different genes of interest was performed using reverse transcription PCR (RT–PCR). Primers were designed using Primer3 combined with BLAST from the NCBI (Table 2).

Quantitative real-time PCR: The quantitative measurement of keratocan, lumican, and α-smooth muscle actin (α-SMA) from different corneal cells was performed with real-time PCR using a DNA Engine Opticon system (MJ Research, Waltham, MA) with the SYBR Green PCR Master Mix (BioRad, Hercules, CA) in a one-step reaction according to the manufacturer's instructions. The primer sequences used are listed in Table 2. The melting curves and gel electrophoresis of the end products were obtained to confirm the specificity of PCR. The relative quantification of target genes was determined using the comparative Ct ($\Delta\Delta^{Ct}$) method.

To measure specific marker mRNA expression, real-time PCR was performed for keratocan, lumican, and α-SMA under the different culture conditions. Keratocytes grown in serum-free medium expressed high levels of keratocan and were keratocan+, lumican+ and α-SMA-, while corneal fibroblasts grown in 10% FBS with 40 ng/ml FGF-2 expressed low levels of all three markers (keratocan, lumican, and α-SMA, Figure 1A). This reinforced the finding that 10% FBS with 40 ng/ml FGF-2 provided the optimal condition for culturing corneal fibroblasts and serum-free DMEM was the optimal condition to grow keratocytes. Quantitative analysis of α-SMA mRNA expression revealed that cells grown in the presence of 2 ng/ml TGF-β1 expressed high levels of α-SMA mRNA and were keratocan-, lumican-, and α-SMA+ (Figure 1A). Expression of keratocan mRNA and α-SMA mRNA was monitored under several other culture conditions (data not shown), and these conditions represented the optimal conditions to culture each of the three primary corneal stromal cell types in vitro (keratocytes, fibroblasts, and myofibroblasts).

Western blot analysis: The cultured cells were trypsinized, and the pellets were extracted in RIPA buffer (Thermo Scientific) containing the protease inhibitor cocktail (Thermo Scientific) at 4 °C for 30 min. The extracts were centrifuged at 15800 ×g for 15 min to remove debris, and the supernatants were used for western blotting to detect α-SMA and β-actin. For keratocan, the cells were extracted by adding 4

mRNA	Up primer 5′ to 3′	Dp primer 5′ to 3′	Amplicon size (bp)
Perlecan	CGTGGCAGTCAACACCAAAG	TTCTTGATGCAGCCCGTGAT	105
Nidogen-1	ATGGTTCAGCATCACTGGG	TAATGACCAGCTTGCCTGGG	80
Nidogen-2	CGTGCAAGACACGGAAGTCA	GAGTTGGCTGGGACGTAAGG	141
Lumican	TGCAGCTTACCCACAACAAG	TGAAGGTGAACGAAGGTCAA	76
Keratocan	AGTGCGGATGACTTTGATTG	TGGCTTCTCTGGAATGGTTT	165
α-SMA	ATTGTGCTATGTCGCTCTGG	GATGAAGGAGGGCTGGAA	157
β-actin	CTGGAACGGTGAAGGTGACA	CGGCCACATTGCAGAACTTT	73

TABLE 2. LIST OF PRIMERS.

M guanidine-HCl containing 10 mM sodium acetate, 10 mM sodium EDTA, 5 mM aminobenzamidine, and 0.1 M ϵ-amino-*n*-caproic acid and protease inhibitor cocktail at 4 °C overnight. The extracts were dialyzed in distilled water, and the precipitates were collected and dissolved in 0.1 M Tris-acetate solution (pH 6.0) containing 6 M urea and protease inhibitor. For keratocan, a 100 µg protein aliquot was incubated with endo-β-galactosidase (0.1 U/ml, Sigma-Aldrich) in 50 mM sodium phosphate (pH 5.8) at 37 °C overnight. After digestion, the protein was lyophilized, dissolved, and underwent western blot analysis for keratocan. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific). Twenty microns of total protein were loaded to each lane on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (8%) for electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk and probed with primary antibodies at 4 °C overnight, α-SMA (1:1,000, M0851, Dako), β-actin (1:5,000, A5441, Sigma-Aldrich), and keratocan (1:500, LS-B8216, LSBio, Seattle, WA). After washing and incubating with corresponding secondary antibodies, protein blotting was visualized by reaction with western horseradish peroxidase substrate (Millipore, Billerica, MA).

To verify the specificity of each cell type, we also monitored the expression of α-SMA and keratocan with western blot. Keratocytes grown in serum-free medium expressed high levels of keratocan compared to cells grown in the presence of serum, FGF-2, and TGF-β1. The cells treated with 10% FBS and FGF-2 had no keratocan expression and was determined to be the best condition for culturing corneal fibroblasts. α-SMA expression was prominent in myofibroblasts (cells grown in the presence of 1% serum and 2 ng/ml TGF-β1; Figure 1B).

Statistical analysis: A sample size of three was used in all experiments. All data are represented as the mean ± SD, and statistical significance was determined using a two-tailed Student *t* test from three or more independent experiments. A p value of less than 0.05 was considered significant.

RESULTS

Analysis of nidogen-1, nidogen-2, and perlecan with real-time PCR: The expression of nidogen-1, nidogen-2, and perlecan was monitored under the three different culture conditions used to culture keratocytes, corneal fibroblasts, and myofibroblasts. Real-time PCR showed nidogen-1 and nidogen-2 mRNAs were highly expressed in keratocytes compared to corneal fibroblasts and myofibroblasts. Conversely, perlecan mRNA was highly expressed in myofibroblasts compared to keratocytes or corneal fibroblasts (Figure 2), although the keratocytes produced significant amounts of perlecan mRNA. Corneal fibroblasts expressed only small amounts of nidogen-1, nidogen-2, and perlecan mRNAs.

Analysis of nidogen-2 and perlecan proteins with immuno-cytochemistry: Nidogen-2 and perlecan protein expression was monitored with immunocytochemistry (Figure 3 and Figure 4). Nidogen-2 localization of expression was different in keratocytes and myofibroblasts. In keratocytes, nidogen-2 appears most highly expressed in a perinuclear organelle, whereas in myofibroblasts the expression was more diffuse and formed a mesh-like network throughout the cells (Figure 3). Staining for nidogen-2 in a perinuclear organelle was also noted in corneal fibroblasts. Perlecan was highly localized
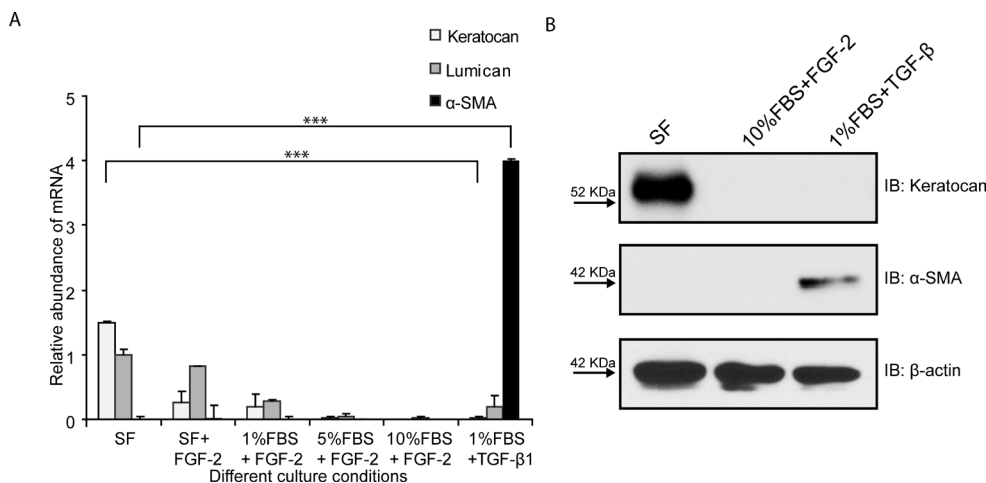

Figure 1. Expression of stromal cell markers under different culture conditions. Cells were cultured in the presence or absence of serum, fibroblast growth factor (FGF)-2, and transforming growth factor (TGF)-β1, as described. SF is serum-free. **A**: Keratocan and lumican mRNA was highly expressed in cells grown in SF medium whereas α-smooth muscle actin (α-SMA) mRNA was highly expressed only in cells grown in the presence of TGF-β1 (n=3; ***, p<0.001; **, p<0.01). **B**: Western blot analysis of keratocan and α-SMA protein expression. As expected, the keratocytes produced keratocan protein, and the myofibroblasts produced α-SMA. The corneal fibroblasts did not produce either marker protein.
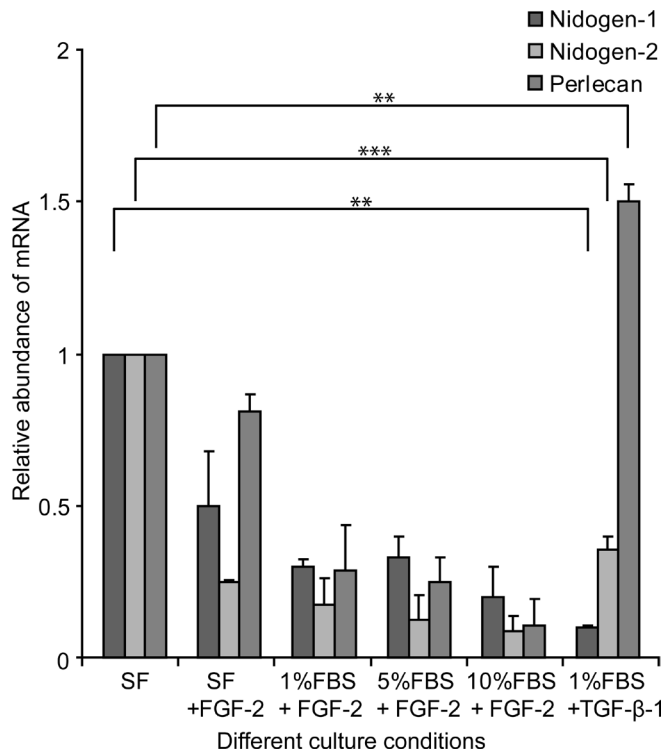
Figure 2. Relative nidogen-1, nidogen-2, and perlecan mRNA levels. Cells were treated under different culture conditions in the presence and absence of serum and growth factors. SF is serum free. Nidogen-1 and nidogen-2 mRNAs were most highly expressed in cells grown in SF medium (keratocytes) whereas perlecan was most highly expressed in cells grown in the presence of transforming growth factor (TGF)-β1 (myofibroblasts; n=3; ***, p<0.001; **, p<0.01).

in what appeared to be intracellular organelles in the keratocytes whereas in the myofibroblasts the perlecan was seen more diffused throughout the cell body (white arrowheads in Figure 4). Fibroblasts had little, if any, detectable expression of perlecan protein. These results show that the levels of expression and localization of expression of nidogen-2 and perlecan are different in the different corneal stromal cell types.

DISCUSSION

The focus of this study was to examine the synthesis of corneal EBM components in different keratocyte-derived corneal stromal cell types present during regeneration of the EBM after injury or surgery that might have a role in defective EBM regeneration in corneas with stromal haze after high-correction PRK [5]. It is important to understand the synthesis of the components involved in EBM formation that could be derived from stromal cells, in addition to the components contributed by the epithelium, which include nidogens and perlecan.

Studies have demonstrated that there is involvement of stromal cells or mesenchymal cells in epithelial basement membrane formation or regeneration. For example, Gipson et al. showed that collagen type VII and laminin are present in the stroma immediately beneath the wounded area at 7 days after keratectomy wounds in rabbit corneas [17]. Studies by Weiser et al. and Thomas and Dziadek have demonstrated the involvement of mesenchymal cells in intestinal and neural basement membrane formation, respectively [18,20]. The present in vitro study was designed to investigate whether the three most common corneal stromal cell types produce EBM components that could contribute to the regeneration of the lamina lucida and lamina densa after corneal injury such as photorefractive keratectomy.

Nidogen-1 and nidogen-2 are closely related sulfated glycoprotein homologs, and both bind to the EBM component laminin [39,40]. In the human cornea, nidogen-2 was found to codistribute with nidogen-1/entactin and is a prominent component of corneal epithelial and limbal vascular BMs [8]. Nidogen to laminin binding has been shown to be the initial step for the bridging of laminin and collagen IV networks [13,15,41-43]. Nidogen-2 interacted with collagens I and IV and perlecan at a comparable level to nidogen-1 but failed to bind to fibulins [44]. The absence of both nidogens completely impaired EBM deposition and structural assembly, while the levels of all other EBM components remained unchanged in the model studied [38]. Perlecan is the most prevalent HSPG in the corneal EBM, and studies by Inomata et al. showed the epithelium to be thin and poorly differentiated in perlecan-deficient mice (Hspg2$^{-/-}$-TG) and accompanied by the downregulation of Ki67, cytokeratin12, connexin43, Notch 1, and Pax6 [12]. These findings suggested that EBM perlecan is critical for the formation and terminal differentiation of the normal corneal epithelium. Given the important roles of nidogens and perlecan in corneal EBM formation and regeneration, including the lamina lucida and lamina densa that are defective in corneas with severe stromal opacity after photorefractive keratectomy [1], the current study used an in vitro model system to study the synthesis of these EBM components by the most common keratocyte-derived corneal stromal cells.

Keratocytes are normally present in the corneal stroma of unwounded corneas, and our results show that the EBM components nidogen-1, nidogen-2, and perlecan are synthesized by keratocytes and myofibroblasts, whereas their localization is different in keratocytes and myofibroblasts in vitro. After surgical wounds such as radial keratectomy stromal incisions or photorefractive keratectomy stromal surface ablation, fibroblasts and myofibroblasts may be generated within

the corneal stroma [45]. Myofibroblasts are also generated from bone marrow–derived cells that migrate into the corneal stroma from the limbal vasculature [46,47]. As stromal wound healing progresses after the wave of keratocyte apoptosis that occurs following injury, keratocytes, fibroblasts, and myofibroblasts may repopulate and survive in the depleted stroma in cellular proportions that depend on the type and extent of the injury, as well as variations in individual animals [48]. Activated keratocytes differentiate into spindle-shaped fibroblasts and then, along with bone marrow-derived fibrocytes, subsequently develop into persistent mature myofibroblasts in the presence of adequate concentrations of epithelium-derived TGF-β1 in the stroma [49-52]. These persistent mature myofibroblasts, and the extracellular matrix they secrete, comprise the anterior stromal opacity associated with persistent "haze" after high ablation photorefractive keratectomy [53,54]. Our working hypothesis is that activated fibroblasts and mature myofibroblasts secrete disorganized extracellular matrix materials but only low levels of EBM components that are not adequate to support normal EBM regeneration, or these cells may interfere with normal EBM regeneration through the production of alternative EBM components. A recent study conducted by Toricelli et al. [19] showed nidogen-2

and perlecan increase in stromal keratocytes after epithelial injury in human cornea. The current study results showed that myofibroblasts produce nidogen-1 mRNA, and nidogen-2 and perlecan mRNA and protein in vitro but that these EBM components localize in a disorganized manner throughout the myofibroblast to form a meshwork-like intracellular pattern. Conversely, in keratocytes nidogen-2 and perlecan are seen predominantly at the cell–cell junction and in a perinuclear organelle. It is not known whether this perinuclear organelle is the nucleolus or another structure. Abnormal localization of EBM components could be an important aspect of stromal cellular contributions to the regenerating nascent EBM. In addition, we have found that rabbit myofibroblasts produce, in addition to the normal sized approximately 150 kDa protein, a prominent, much larger (greater than 250 kDa) nidogen-2 protein, not produced by keratocytes or corneal fibroblasts, when cellular proteins are analyzed with western blotting (Santhanam and Wilson, unpublished data, 2015). Ongoing work is aimed at characterizing this alternative protein. Corneal fibroblasts express small amounts of nidogens or perlecan, which is an interesting finding since corneal fibroblasts are likely the intermediate cell type between keratocytes and myofibroblasts when the latter cells are derived
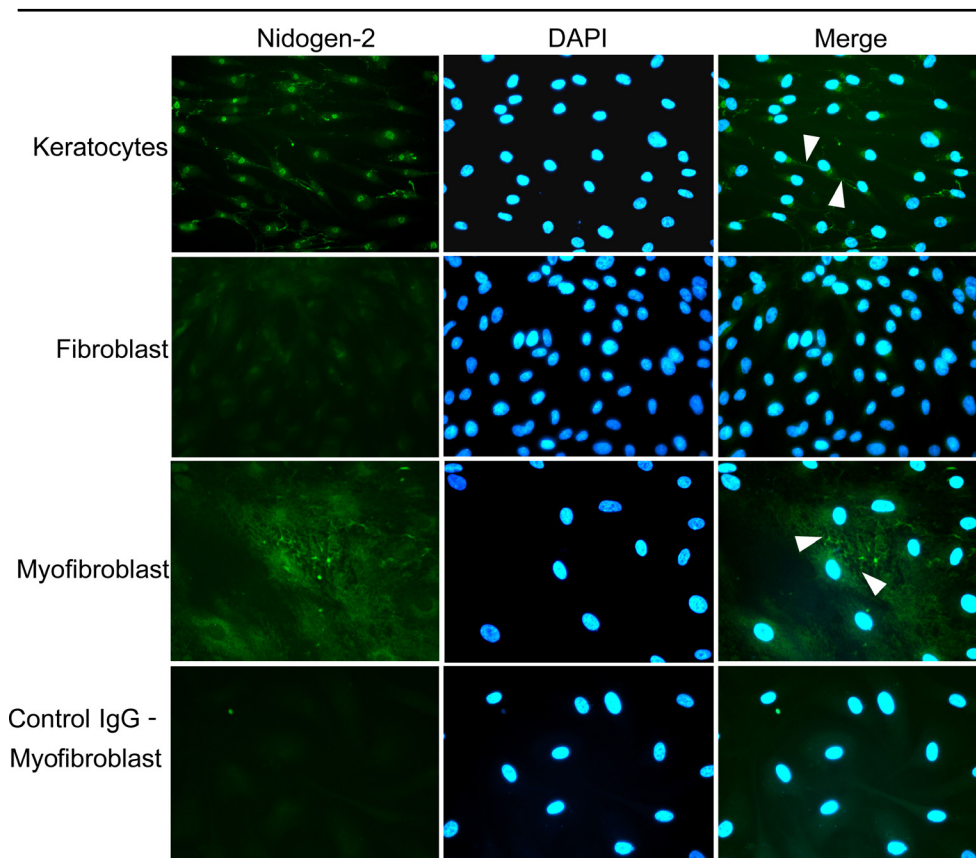

Figure 3. Nidogen-2 immunocytochemistry of corneal stromal cells under different culture conditions. The expression of nidogen-2 protein (green) is seen to be highest in the perinuclear organelle, whereas in myofibroblasts nidogen-2 protein expression appears as a meshwork-like pattern throughout the cell body (white arrowheads). Fibroblasts have little, if any, nidogen-2 protein expression, except the perinuclear organelle staining, as was observed in keratocytes. Isotypic control immunoglobulin (IgG) staining was negative under all cell conditions, as shown for the myofibroblasts. Magnification=400X.

from corneal stromal cells instead of bone marrow–derived cells [55]. Thus, qualitative and quantitative differences in expression of EBM proteins, localization of these proteins, or other as yet unidentified factors could be important determinates of defective EBM regeneration in corneas with haze after photorefractive keratectomy or other injuries. Further study is needed to characterize stromal cell contributions to EBM regeneration after corneal injury to understand the pathophysiology of corneal stromal opacity after injury, surgery, and infection.

APPENDIX 1. MORPHOLOGY OF CELLS UNDER DIFFERENT CULTURE CONDITIONS.

Cells in panel A were treated with serum-free DMEM medium and panel B cells were treated with serum-free DMEM (SF) with 40 ng/ml FGF-2. Cells in panel C, D, E are treated with DMEM containing 1%, 5%, 10% FBS with 40 ng/ml FGF-2, respectively. Heparin sulfate (5 µg/ml) was always added along with FGF-2. Cells in panel F are treated with DMEM containing 1% FBS with 2 ng/ml TGF-β. The difference in the morphology of cells grown in the presence and absence of serum and growth factors can be noted.

Mag. 100X. To access these data, click or select the words "Appendix 1".

APPENDIX 2. A-SMA IMMUNOCYTOCHEMISTRY OF CELLS UNDER DIFFERENT CULTURE CONDITIONS.

Cells in panel A were treated with serum-free DMEM medium and cells in panel B were treated with serum-free DMEM with 40 ng/ml FGF-2 and 5 µg/ml heparin sulfate. Cells in panel C were treated with DMEM containing 1% FBS and 2 ng/ml TGF-β. α-SMA (red) is seen in cells grown in the presence of TGF-β but not in the cells grown under the other conditions. DAPI is blue. Mag. 400X. To access these data, click or select the words "Appendix 2".
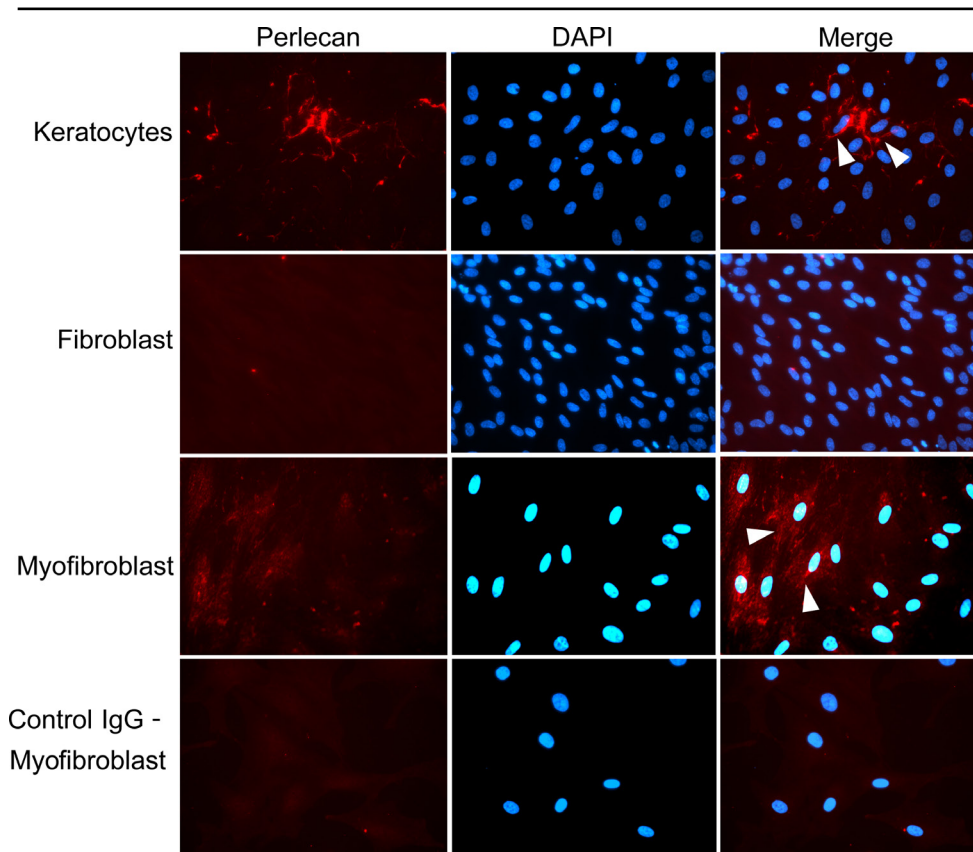
Figure 4. Perlecan immunocytochemistry of corneal stromal cells cultured under different conditions. Perlecan (red) was most highly expressed in localized intracellular structures in keratocytes whereas in the myofibroblasts perlecan was detected more diffusely throughout the cell (white arrowheads). Fibroblasts had little, if any, detectable expression of perlecan protein. The isotypic control immunoglobulin (IgG) did not yield staining under any of the culture conditions, as is shown for myofibroblasts. Magnification=400X.

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