Quantitative proteomic comparison of myofibroblasts derived from bone marrow and cornea

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Myofibroblasts are fibroblastic cells that function in wound healing, tissue repair and fibrosis, and arise from bone marrow (BM)-derived fibrocytes and a variety of local progenitor cells. In the cornea, myofibroblasts are derived primarily from stromal keratocytes and from BM-derived fibrocytes after epithelial-stromal and endothelial-stromal injuries. Quantitative proteomic comparison of mature alpha-smooth muscle actin (α-SMA)+ myofibroblasts (verified by immunocytochemistry for vimentin, α-SMA, desmin, and vinculin) generated from rabbit corneal fibroblasts treated with transforming growth factor (TGF) beta-1 or generated directly from cultured BM treated with TGF beta-1 was pursued for insights into possible functional differences. Paired cornea-derived and BM-derived α-SMA+ myofibroblast primary cultures were generated from four New Zealand white rabbits and confirmed to be myofibroblasts by immunocytochemistry. Paired cornea- and BM-derived myofibroblast specimens from each rabbit were analyzed by LC MS/MS iTRAQ technology using an Orbitrap Fusion Lumos Trubrid mass spectrometer, the Mascot search engine, the weighted average quantification method and the UniProt rabbit and human databases. From 2329 proteins quantified with ≥2 unique peptides from ≥3 rabbits, a total of 673 differentially expressed (DE) proteins were identified. Bioinformatic analysis of DE proteins with Ingenuity Pathway Analysis implicate progenitor-dependent functional differences in myofibroblasts that could impact tissue development. Our results suggest BM-derived myofibroblasts may be more prone to the formation of excessive cellular and extracellular material that are characteristic of fibrosis.

Myofibroblasts are cells that have indispensable roles in normal wound healing and tissue repair in all organs1 and the development and persistence of these cells is central to the pathophysiology of fibrosis2,3. Myofibroblasts may develop from fibroblasts4, fibrocytes5,6, epithelial cells or endothelial cells through mesenchymal transition7,8 or Schwann cells9, and possibly from other cells, depending on the location and type of injury, the genetic makeup of the individual animal and other unknown factors. In the cornea, for example, depending on the injury, the species of animal, and even the individual animal, myofibroblasts develop from keratocytes—via corneal fibroblasts—or fibrocytes in response to epithelial-stromal or endothelial-stromal injury, and resulting ongoing penetration of TGFβ into the corneal stroma at sufficient levels to drive myofibroblast development10. Keratocytes transition to corneal fibroblasts—which then differentiate into α-smooth muscle actin (α-SMA)-expressing myofibroblasts11,12. However, it has also been shown that infiltrating fibrocytes differentiate into myofibroblasts in mice after scar-producing epithelial-stromal injuries to the cornea using chimeric mice expressing green fluorescent protein and immunohistochemistry for fibrocyte and myofibroblast markers6,13. Mature myofibroblasts secrete different collagens and other extracellular matrix materials that make up the scars that are characteristic of fibrosis1.
Many studies have been performed to identify the progenitor cells for myofibroblasts in lung, liver and other organs\(^{4,5,15}\), but little is known about differences in function, if any, between myofibroblasts that arise from local progenitors compared to BM-derived progenitors. This study pursued quantitative proteomic comparison of rabbit myofibroblasts using LC MS/MS Isobaric Tag for Relative and Absolute Quantification (iTRAQ) technology for further insights into whether myofibroblasts that differentiate from corneal keratocytes are functionally different from myofibroblasts that differentiate from BM-derived cells.

**Materials and methods**

**Animals.** All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Institutional Animal Control and Use Committee at the Cleveland Clinic approved these studies. Four 12 to 15 week old female New Zealand white rabbits that weighed 2.5 to 3.0 kg were obtained from Charles River Laboratories, MA, USA.

**Myofibroblast primary cell cultures.** Four separate primary keratocyte-derived corneal fibroblast cultures were generated from both eyes of the four New Zealand white rabbit corneas, as previously described\(^{16}\) with Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, NY). Briefly\(^{16}\), primary keratocytes were isolated from each cornea by first removing the epithelial and endothelial layers 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 by digestion 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) with 1% FBS and 20 ng/ml TGF-β1 (R&D, Minneapolis, MN). The medium was changed every 48 h in all cultures.

BM-derived myofibroblast primary cultures were generated from the same four rabbits as described previously to generate cornea-derived myofibroblasts. Briefly, \(13 = \) the tibias and femurs of rabbits were removed and BM cells were harvested by flushing medium and scratching the bone marrow cavity with the end of an 18-gauge needle. BM cells were collected in a petri dish and clumps were teased out and gently dissociated with a one ml pipette to form a single-cell suspension. The suspension of cells was centrifuged at 1500 rpm for 10 min at 4 °C to obtain a cell pellet. Red blood cells were lysed by adding sterile Milli-Q water at 4 °C, followed by dilution with 10X PBS at 4 °C at a ratio of one part PBS to nine parts cell solution, with immediate mixing. Cell suspensions were centrifuged again at 1500 rpm for 10 min at 4 °C and re-suspended in 1 × PBS at 4 °C. Cell viability in the range of 90% to 95% was verified by staining with 0.4% trypan blue. Cells were suspended in PBS at 4 °C at a final concentration of 2 × 10⁶ cells/ml. BM-derived cells were cultured in DMEM (Gibco) with 1% FBS and 20 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN). The medium was changed every 48 h in all cultures.

Cornea- and BM-derived myofibroblast cultures were each harvested after 14 days of culture with 20 ng/ml TGF-β1, the cells washed with PBS, and cell pellets frozen at −80 °C until analysis.

Cornea- and BM-derived myofibroblast cultures were grown in parallel in Nunc Lab-tek 8-well chamber slides (#154534, Thermo Fisher Sci, Waltham, MA), washed twice in PBS, and after fixation with IC Fixation Buffer (#00-8222-49 Thermo Fisher Scientific) for 10 min, washing twice with PBS and incubated for 1 h with 5% donkey serum. Immunocytochemistry was performed for myofibroblast-related markers by immersing cell layers in antibodies to α-SMA (M0851, DAKO, Glostrup, Denmark, 1:100), vincinin (Mab2105, R&D Systems, Minneapolis, MN, 1:50), desmin (D1033, Millipore Sigma, St Louis, MO, 1:40), or vinculin (MAB3574, Millipore Sigma, 1:100) in PBS for 1 h. Control immunocytochemistry in both cornea- and BM-derived myofibroblasts was performed by substituting isotypic control antibodies as the primary antibody (mouse IgG2a kappa cat#01-675-858, mouse IgG1 cat#02-610-0, or rat IgG2a cat#02-968-8 from Invitrogen, Carlsbad, CA). Slides were washed twice with PBS and incubated for 1 h in the corresponding Alexa Fluor (Thermo Fisher Scientific) secondary antibodies at 1:200 in PBS. Slides were washed 3 times in PBS before application of DAPI and a coverslip sealed with nail polish. Slides were analyzed and imaged with a Leica DM5000 microscope (Leica, Buffalo Grove, IL, USA) equipped with Q-imaging Retiga 4000RV (Surrey, BC, Canada) camera and Image-Pro software (Media Cybernetics Inc., Bethesda, MD, USA).

**Sample preparation.** Individual pellets from each of the cornea- and BM-derived myofibroblast preparations were homogenized in 100 mM triethylammonium bicarbonate containing 2% SDS, the protein extracted three times from the cell debris and quantified by AccQ-Tag amino acid analysis\(^{17}\). Approximately 290 µg protein per rabbit was recovered from the corneal myofibroblast cultures and 850 µg protein per rabbit from the BM myofibroblast cultures. Soluble myofibroblast protein (100 µg) from each of the specimens was reduced with tris-(2-carboxyethyl) phosphine, cysteines alkylated with methyl methanethiosulfonate, then the protein was precipitated with acetone overnight. Protein pellets were washed twice with ice cold 67% acetone, gently blown-dry with argon and re-suspended in 50 mM M Tris-Cl, and digested overnight at 37 °C with trypsin (initially with 2% trypsin (w/w), followed in 2 h with another 2% (w/w), and the next day with another 1% (w/w) for 2 h additional incubation). Following proteolysis, soluble peptides were quantified by AccQ-Tag amino acid analysis.

**ITRAQ labeling and peptide fractionation.** ITRAQ labeling with an 8-plex iTRAQ kit were performed as previously described\(^{19-21}\). In this study, digestive tryptic digests of each myofibroblast preparation (100 µg/specimen) were labeled individually with a different iTRAQ tag and the labeled specimens mixed together in equal amounts and fractionated by reverse phase high performance liquid chromatography (RP-HPLC) at pH 10 on a Waters xBridge BEH300 C18 column (3.5µ particle size, 2.1 × 100 mm). Chromatography was performed at a flow rate...
of 200 µL/min using aqueous acetonitrile/0.1% NH₄OH solvents, a 0.5%/min acetonitrile gradient over 50 min; absorbance was monitored at 214 nm and fractions were collected at 1 min intervals. Chromatography fractions encompassing the entire elution were selectively combined, dried, and a total of 17 fractions were analyzed by LC MS/MS.

Protein identification. RPHPLC pH10 chromatography fractions were analyzed by LC MS/MS with an Orbitrap Fusion Lumos Trividir mass spectrometer27–28. Protein identification utilized the Mascot 2.6.2 search engine, and the Uniprot rabbit reference proteome database version 20190730 (21,264 sequences, 982 reviewed and 20,282 unreviewed) and the Uniprot human reference proteome database version 20190730 (96,464 sequences, 42,412 reviewed and 54,052 unreviewed). The Uniprot rabbit database is currently incomplete therefore protein identification utilized both the rabbit and human databases as rabbits are closely related phylogenetically to primates23. Proteins were identified in four categories including: (1) proteins characterized only in the rabbit database; (2) proteins characterized in both the rabbit and human databases; (3) proteins uncharacterized in the rabbit database but characterized in the human database; and (4) proteins characterized only in the human database. Sequence Identity between identified rabbit and human proteins was determined using Blast 2.9.023.

Protein identification required detection of a minimum of two unique peptides per protein and a database gene symbol. Database search parameters were restricted to three missed tryptic cleavage sites, a precursor ion mass tolerance of 10 ppm, a fragment ion mass tolerance of 20 mmu and a false discovery rate of ≤1%. Fixed protein modifications included N-terminal and ε-Lys iTRAQ modifications and S-methyl-Cys. Variable protein modifications included Met oxidation, Asn and Gin deamidation and iTRAQ Tyr. A minimum Mascot ion score of 25 was used for accepting peptide MS/MS spectra.

Protein quantitation. iTRAQ tags were quantified by the weighted average method24 using the Mascot 2.6.2 Summed Intensities program. Protein quantitation required a minimum of two unique peptides per protein, Mascot peptide ion scores ≥ 25, and utilized reporter ion tolerance of 10 ppm. Protein ratios were determined in log space and transformed for reporting. Proteins exhibiting average protein ratios above or below the mean by at least 1 standard deviation and p values ≤ 0.05 (pairwise moderated t-test adjusted for multiple testing) were considered significantly elevated or decreased.

Statistics and bioinformatics. Programs available in R25, including the Limma package26, were used for normalizing the protein ratios to quantiles, determining standard error of the mean (SEM) and p values (moderated t-test) from paired samples with adjustment for multiple testing and for identifying differentially expressed (DE) proteins27. For average results, calculation of SEM and adjusted p values required ≥ 3 samples. Bioinformatic analyses were performed with Ingenuity Pathways Analysis (Qiagen).

Western blot analysis. Western blot analysis of cornea-derived and BM-derived myofibroblasts was performed using a previously detailed method26 with a 7.5% acrylamide precast gels (Bio-Rad, Hercules, California) and enhanced chemiluminescense for signal detection (GE, Life Sciences, Marlborough, MA). Celluar protein (10 µg) was resolved on the 4–15% SDS-PAGE gels then transferred to PVDF membranes for immunoblotting. The membranes were blocked with 5% non-fat milk and probed with primary antibodies at 4 °C overnight. Primary antibodies were anti-collagen III (cat. no. ARG20786, Arigo Bio, Cedar lane NC, USA, 1:500 dilution), anti-collagen XI (cat no. LS-C151380, LS Bio, USA, 1:1000 dilution), anti-collagen VII (cat no. ab223639, Abcam, USA, 1:5000 dilution) and anti-β actin (cat. no. A5441, SIGMA, USA, 1:5000 dilution). Secondary antibodies (donkey anti-mouse –HRP, cat no. sc2314; donkey anti-goat –HRP, cat no. sc202) were obtained from Santa Cruz Biotechnology (Dallas, TX) and used at 1:10,000 dilution. Western blot signal intensities were quantified by densitometry using Image J software (NIH, Bethesda, MD).

Results

Overview. Cornea and BM were isolated from four New Zealand white rabbits and four myofibroblast primary cultures were generated from each tissue. Myofibroblast identity and homogeneity were confirmed by immunocytochemistry (Fig. 1) and paired cornea- and BM-derived myofibroblast specimens were analyzed by LC MS/MS iTRAQ technology, yielding a total of 2420 proteins quantified, of which 2329 were quantified in ≥ 3 rabbits. Proteomic results are summarized in Table 1 and presented in detail for each rabbit in Supplemental Tables S1–S4. These results include protein accession numbers and descriptions, gene symbols, protein ratios (cornea/BM), number of unique peptides, number of summed peptide intensities, percent sequence coverage, database identification category, and percent identity between rabbit and human proteins. The proteomic results from all four paired samples were similar in quality and exhibited near-to-normal distributions (Fig. 2) and therefore the quantification was suitable for averaging. The mean relative abundance of proteins quantified in all the myofibroblast samples is presented in Supplemental Table S5, including sample frequency, standard error of the mean, and moderated p values adjusted for multiple testing. A total 673 DE proteins were identified, as illustrated by Volcano plot (Fig. 3) and itemized in Supplemental Table S5 with color coding. Criteria for identifying DE proteins included: (i) quantification in ≥ 3 of the paired myofibroblast samples; (ii) an average protein ratio above or below the mean by at least 1 standard deviation (SD), and (iii) an average ratio with an adjusted p value ≤ 0.05.

Proteomic comparison of myofibroblasts from cornea or BM. Comparative proteomic analysis of myofibroblasts was performed with 2329 proteins quantified in ≥ 3 of the four paired cornea and BM myofibro-
blast samples. Three hundred sixty proteins (~15%) were found significantly more abundant in cornea- than BM-derived myofibroblasts and are considered to be differentially expressed (Table S5). Thirty-three of these 360 proteins exhibited ratios ≥ 2 SD from the mean. Three hundred thirteen DE proteins were found significantly more abundant in BM- than cornea-derived myofibroblasts (Table S5), including 63 proteins exhibiting ratios ≥ 2 SD from the mean. Proteomic differences between these two types of myofibroblasts are suggested by a comparison of 30 proteins that are significantly more abundant in cornea- (Table 2) or BM- (Table 3) derived progenitor cells. Bioinformatic comparison of all DE proteins more abundant in either the cornea-derived myofi-
Figure 2. Distribution of myofibroblast protein ratios. The LN mean distribution of myofibroblast protein ratios (cornea/BM) are shown for proteins quantified in paired samples from four different rabbits. The histogram represents a total of 2420 proteins quantified with ≥ 2 unique peptides. LN median = 0, LN Mean = 0, and SD = 0.38. The distribution of protein ratios is near-to-normal and statistically appropriate for comparing the proteomes of myofibroblasts derived from cornea and BM.

Figure 3. Differentially expressed myofibroblast proteins. This volcano plot shows LN average protein ratios (cornea/BM) versus $p$ values (paired t-test adjusted for multiple testing) for 2329 proteins quantified in ≥ 3 paired myofibroblast specimen. Vertical dashed lines represent protein fold changes above and below the mean by 1 standard deviations (SD), with proteins above the horizontal line ($p$ values < 0.05) considered differentially expressed.
Table 2. Differentially expressed proteins proteins significantly more abundant in rabbit myofibroblasts from cornea than from BM. The above 30 proteins were selected from 360 differentially expressed proteins more abundant in rabbit myofibroblasts from cornea than from BM. Each exhibited a protein ratio ≥ 1 SD from the mean and an adjusted p values ≤ 0.05 in ≥ 3 paired myofibroblast samples. All differentially expressed proteins are illustrated in Fig. 2 and identified in Supplemental Table 5.

| Rabbit accession | Human accession | Gene symbol | Protein | Sample frequency | Linear ratio cornea/bone marrow | % identity rabbit and human proteins | Adjusted p value
|------------------|----------------|-------------|---------|-----------------|-------------------------------|-------------------------------------|-------------------|
| G15W85           | Q92820         | GGH         | Folate gamma-glutamyl hydrolase | 4                | 4.78             | 82                    | 3.5E−04           |
| G1U4P8           | Q9BXN1         | ASPN        | Asporin | 4                | 3.05             | 88                    | 3.5E−04           |
| G15DA2           | P29762         | CRABP1      | FABP domain-containing Cellular retinoic acid-binding protein 1-like protein | 4                | 2.89             | 99                    | 1.7E−04           |
| G1TW43           | J3QSU6         | TNC         | Tenascin | 4                | 2.73             | 74                    | 1.7E−04           |
| G1ST69           | P20709         | LMNB1       | Lamin-B1 | 4                | 2.73             | 98                    | 1.7E−04           |
| G1T380           | Q02388         | COL7A1      | Collagen alpha-1(VII) chain | 4                | 2.73             | 87                    | 1.6E−03           |
| G1SIJF4          | A0A0A0MR51     | FADS1       | Acyl-CoA (8–3)-desaturase | 4                | 2.66             | 93                    | 4.7E−04           |
| G1T3Y8           | P10809         | HSPD1       | 60 kDa heat shock protein, mitochondrial | 4                | 2.66             | 99                    | 1.7E−04           |
| G1SJW7           | J3QLE5         | SNRPN       | Small nuclear ribonucleoprotein-associated protein | 4                | 2.57             | 100                   | 1.7E−04           |
| G1SK42           | P21980         | TGM2        | Protein-glycine gamma-glutamyltransferase 2 | 4                | 2.55             | 87                    | 2.8E−04           |
| G1SEF5           | Q8INAS         | PRPF38A     | Pre-mRNA-splicing factor 38A | 4                | 2.52             | 100                   | 3.3E−04           |
| G1SNX3           | B4DY10         | ILF2        | Interleukin enhancer-binding factor 2 | 4                | 2.45             | 100                   | 1.7E−04           |
| G1T20X           | Q90541         | FLN2        | Perilipin-2 | 4                | 2.45             | 88                    | 2.6E−03           |
| G1SIJF9          | P51991         | HNRNPAA3    | Heterogeneous nuclear ribonucleoprotein A3 | 4                | 2.44             | 100                   | 1.7E−04           |
| G1SFC6           | A0A087WV29     | NAT10       | RNA cytidine acetyltransferase | 4                | 2.41             | 96                    | 2.2E−04           |
| G1TD28           | D6RRA6         | TMEM33      | Transmembrane protein 33 (Fragment) | 4                | 2.40             | 99                    | 1.7E−04           |
| G1SUP9           | P40087         | NOP2        | Probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase | 4                | 2.40             | 77                    | 1.9E−04           |
| P00389           | P16435         | POR         | NADPH-cytochrome P450 reductase | 4                | 2.37             | 92                    | 2.5E−04           |
| G1TLW3           | J3KTA4         | DDX5        | Probable ATP-dependent RNA helicase DDX5 | 4                | 2.35             | 96                    | 1.7E−04           |
| G1SDW8           | H3Y2P0         | CD44        | CD44 antigen (Fragment) | 4                | 2.34             | 91                    | 5.2E−04           |
| G1T4M2           | O60264         | SMARCA5     | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 | 4                | 2.321            | 100                   | 2.5E−04           |
| G1T2K5           | J3L1L3         | MYBBP1A     | Myb-binding protein 1A (Fragment) | 4                | 2.318            | 69                    | 1.9E−04           |
| G1SI26           | Q4VC31         | CCD58       | Coiled-coil domain-containing protein 58 | 4                | 2.277            | 95                    | 1.7E−04           |
| G1TX84           | Q9Y5J1         | UTP18       | U3 small nuclear RNA-associated protein 18 homolog | 4                | 2.269            | 89                    | 1.7E−04           |
| G1SMR3           | Q9NV31         | IMP3        | U3 small nuclear ribonucleoprotein protein IMP3 | 4                | 2.260            | 99                    | 1.7E−04           |
| G1SE74           | Q7KZ65         | SPT6H       | Transcription elongation factor SPT6 | 4                | 2.247            | 99                    | 2.8E−04           |
| G1T3Y0           | P90802         | DAB2        | Disabled homolog 2 | 4                | 2.233            | 90                    | 3.3E−04           |
| G1SI36           | Q13308         | PTK7        | Inactive tyrosine-protein kinase 7 | 4                | 2.232            | 93                    | 1.7E−04           |
| G1TA41           | G0ILb6         | HNRNPH1     | Heterogeneous nuclear ribonucleoprotein H | 4                | 2.226            | 98                    | 1.7E−04           |
| G1T6T0           | J3RKNJ3        | NAALAD2     | N-acetylated alpha-linked dipeptidase 2 | 4                | 2.215            | 89                    | 1.9E−04           |
compared to BM-derived myofibroblasts, respectively.

as most predominant in BM-derived cells (Table 5). Molecular and cellular functions of cornea- and BM-derived

cornea after injuries28. Conversely, since corneal keratocyte-derived myofibroblasts produce more collagen type
broblasts produce much more collagen type XI and collagen type III, they likely contribute greatly to structure

involved in fibrosis mediated by myofibroblasts (collagen type III, collagen type VII and collagen type XI.) Cons-
istent with the iTRAQ quantitation, immunoblot results confirmed that collagen type III and collagen type
XI were decreased (Fig. 4A, B) and collagen type VII was elevated (Fig. 4C) in cornea-derived myofibroblasts
compared to BM-derived myofibroblasts, respectively.

Independent evidence supporting the iTRAQ protein quantitation. Western blot analysis was

in this study were at similar stages of myofibroblast differentiation as cell culture procedures were
carefully coordinated and 100% of the cells in all cultures were α-SMA+16.

Discussion

Myofibroblasts are critical mediators of fibrosis that may occur in most organs of animals after injury4. Typically,
at least two progenitor cells to myofibroblasts have been found to participate in the pathophysiology of fibrosis
in each organ evaluated7–9,13. In the present study, we have compared the proteome of corneal keratocyte-derived
myofibroblasts to that of BM-derived myofibroblasts—both of which have been shown to be major progenitors
to myofibroblasts in fibrosis that occurs after corneal injury6,13. The keratocyte-derived and BM-derived myofi-
broblasts used in this study were at similar stages of myofibroblast differentiation as cell culture procedures were

The present study used quantitative proteomics technology to identify DE proteins in myofibroblasts derived
from cornea and BM progenitors. The data indicates about 29% of the proteins quantified were differentially
expressed between these two types of myofibroblasts. Proteomic differences were confirmed by Western blot
analysis of three proteins likely to be involved in the pathophysiology of fibrosis, namely collagen type III,
collagen type VII and collagen type XI. Clues to progenitor-dependent differences in myofibroblasts were sug-
gested by bioinformatic analysis of the DE proteins. Canonical pathways involving mitochondrial dysfunction,
oxidative phosphorylation and sirtuin signaling were implicated as most predominant in cornea-derived cells
(Table 4), and pathways involving glycolysis I, integrin signaling and remodeling of epithelial adherens junctions

Top functions of DE proteins more abundant in BM-derived myofibroblasts were
bioinformatically centered on cellular organization, degranulation of cells, microtubule dynamics, fibrogenesis,
cell movement, and formation of filaments and cell protrusions (Table 5). For example, since BM-derived myofi-
broblasts produce much more collagen type XI and collagen type III, they likely contribute greatly to structure
and strength of fibrotic tissue in the cornea and may contribute most of the collagen type III deposited in the

cornea after injuries28. Conversely, since corneal keratocyte-derived myofibroblasts produce more collagen type

The bioinformatic results support the likelihood of progenitor-dependent functional differences in myofibro-
blasts, however, potentially limiting factors warrant acknowledgement. These factors include the relatively small
sample size employed, namely only four rabbits, and the fact that the rabbit protein sequence databases are not
well developed and are incompletely curated. Nevertheless, rabbits are close phylogenetic relatives of humans22
and utilization of the human UniProt sequence database, one of the most complete and well-curated databases
available, supports the reliability of the protein identifications. Notably, the overall level of significant proteomic
differences (~ 29%) observed between cornea- and BM-derived myofibroblasts is 2–3× greater than the variability
reported from repetitive proteomic analysis of a variety of normal tissues19,23. Furthermore, the levels of three
different collagens thought to contribute to corneal fibrosis, were confirmed to be different between keratocyte-
derived myofibroblasts and BM-derived myofibroblasts. It’s possible that the BM-derived myofibroblasts analyzed
in this study could have been heterogeneous if they were derived from both bone marrow fibrocytes and bone
marrow stromal cells after stimulation with TGF beta-132,33. Another possible limitation is that these two types of
myofibroblasts were cultured and characterized in vitro, and it is not certain that the cells generated in vivo
and in vitro are the same. However, the expression of vimentin, α-SMA and desmin found for both myofibroblast
types in vitro was similar to findings after fibrosis-producing injuries in situ14.

The results of this study suggest that the myofibroblasts derived from different progenitors contribute dif-
ferentially, and perhaps additively, to the fibrosis response to injury in the cornea. This further suggests that the
character of the fibrotic tissue may vary depending on the relative contributions of the myofibroblast progenitors.
For example, after anterior corneal injury produced by irregular phototherapeutic keratectomy (PTK) to inhibit
epithelial basement membrane regeneration in mice, 30 to 70% of myofibroblasts were derived from BM-derived
progenitors, with the remaining myofibroblasts developed from keratocyte-derived progenitors6, although some
possibly developed from Schwann cells57. How the variation in myofibroblasts would affect properties of fibrosis
such as contractility, opacity or persistence in the cornea remains unknown. A recent in vitro study36 showed
that the numbers of α-smooth muscle actin+ myofibroblasts generated from either keratocyte-derived pre-
cursors or BM-derived precursor cells were higher when both cells were co-cultured together in a culture flask

The present study used iTRAQ protein quantitation to independently corroborate the iTRAQ protein quantitation by analysis of three proteins likely to be involved in fibrosis mediated by myofibroblasts (collagen type III, collagen type VII and collagen type XI). Consistent with the iTRAQ quantitation, immunoblot results confirmed that collagen type III and collagen type XI were decreased (Fig. 4A, B) and collagen type VII was elevated (Fig. 4C) in cornea-derived myofibroblasts compared to BM-derived myofibroblasts, respectively.

Independent evidence supporting the iTRAQ protein quantitation. Western blot analysis was used to independently corroborate the iTRAQ protein quantitation by analysis of three proteins likely to be involved in fibrosis mediated by myofibroblasts (collagen type III, collagen type VII and collagen type XI). Consistent with the iTRAQ quantitation, immunoblot results confirmed that collagen type III and collagen type XI were decreased (Fig. 4A, B) and collagen type VII was elevated (Fig. 4C) in cornea-derived myofibroblasts compared to BM-derived myofibroblasts, respectively.

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Table 3. Differentially expressed proteins significantly more abundant in rabbit myofibroblasts from BM than from cornea. The above 30 proteins were selected from 313 differentially expressed proteins more abundant in rabbit myofibroblasts derived from BM than from cornea. Each exhibits a protein ratio ≥ 1 SD from the mean and an adjusted p values ≤ 0.05 in ≥ 3 paired myofibroblast samples. All differently expressed proteins are illustrated in Fig. 2 and identified in Supplemental Table 5.
(juxtacrine) as compared to when BM-derived precursor cells and keratocyte-derived precursor cells were co-culture in different compartments of a Transwell System (paracrine). This suggests that the presence of the two different myofibroblasts cells in the stroma after injury may potentiate the overall fibrosis response. Our current proteomic and bioinformatic results suggest that BM-derived myofibroblasts may be more prone than cornea-derived precursors to impact cellular organization and the formation of excessive cellular and extracellular material characteristic of fibrosis. Hopefully, the findings of this study will stimulate future research to better understand the contributions of myofibroblasts from different precursors to the fibrosis response, and toward the development of more effective therapies to fibrotic tissue damage.

**Table 4.** Bioinformatic analyses of proteins more abundant in cornea-derived myofibroblasts. Bioinformatic properties of 360 differentially expressed proteins more abundant in cornea- than BM-derived myofibroblasts were determined using Ingenuity Pathway Analysis. BH $p$ value refers to the false discovery rate.

| BH $p$ value | Top canonical pathways |
|--------------|------------------------|
| $1.9\times10^{-10}$ | Mitochondrial dysfunction |
| $3.9\times10^{-09}$ | Oxidative phosphorylation |
| $5.0\times10^{-08}$ | Sirtuin signaling pathway |

| BH $p$ value | Top molecular and cellular functions |
|--------------|-----------------------------------|
| $5.1\times10^{-74}$ | Processing of RNA |
| $3.4\times10^{-56}$ | Splicing of mRNA |
| $3.2\times10^{-16}$ | Transport of mRNA |
| $4.8\times10^{-16}$ | Export of mRNA |
| $3.6\times10^{-14}$ | Translation |
| $3.6\times10^{-13}$ | Translation of protein |
| $8.7\times10^{-11}$ | Homologous recombination of cells |
| $8.1\times10^{-07}$ | DNA recombination |

**Table 5.** Bioinformatic analyses of proteins more abundant in BM-derived myofibroblasts. Bioinformatic properties of 313 differentially expressed proteins more abundant in BM- than cornea-derived myofibroblasts were determined using Ingenuity Pathway Analysis. BH $p$ value refers to the false discovery rate.

| BH $p$ value | Top canonical pathways |
|--------------|------------------------|
| $7.9\times10^{-09}$ | Actin cytoskeleton signaling |
| $7.9\times10^{-09}$ | Glycolysis I |
| $1.6\times10^{-08}$ | Integrin signalling |
| $1.6\times10^{-08}$ | Remodeling of epithelial adherens junctions |

| BH $p$ value | Top molecular and cellular functions |
|--------------|-----------------------------------|
| $4.2\times10^{-24}$ | Organization of cytoplasm |
| $7.1\times10^{-21}$ | Degranulation of cells |
| $1.3\times10^{-20}$ | Organization of cytoskeleton |
| $4.3\times10^{-15}$ | Microtubule dynamics |
| $4.3\times10^{-15}$ | Fibrogenesis |
| $1.0\times10^{-14}$ | Formation of filaments |
| $1.5\times10^{-12}$ | Cell movement |
| $4.7\times10^{-11}$ | Formation of cellular protrusions |
Data generated during this study are included as Supplemental Tables S1–S5 and as Supplemental Figure S1. The original mass spectra are publicly available from MassIVE (https://massive.ucsd.edu) using the identifier MSV000084599.

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