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Keratocan, a Cornea-specific Keratan Sulfate Proteoglycan, Is Regulated by Lumican*

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Abstract

Lumican is an extracellular matrix glycoprotein widely distributed in mammalian connective tissues. Corneal lumican modified with keratan sulfate constitutes one of the major proteoglycans of the stroma. Lumican-null mice exhibit altered collagen fibril organization and loss of corneal transparency. A closely related protein, keratocan, carries the remaining keratan sulfate of the cornea, but keratocan-null mice exhibit a less severe corneal phenotype. In the current study, we examined the effect of lumican overexpression in corneas of wild type mice. These mice showed no alteration in collagen organization or transparency but had increased keratocan expression at both protein and mRNA levels. Corneas of lumican-null mice showed decreased keratocan. This coupling of keratocan expression with lumican also was observed after intrastromal injection of a lumican expression minigene into the corneal stroma of *Lum*$^{-/-}$ mice. Small interfering RNA knockdown of lumican *in vitro* reduced keratocan expression, whereas co-injection of a lumican-expressing minigene with a *β*-galactosidase reporter driven by the keratocan promoter demonstrated an increase of keratocan transcriptional activity in response to lumican expression in *Lum*$^{-/-}$ corneas *in vivo*. These observations demonstrate that lumican has a novel regulatory role in keratocan expression at the transcriptional level. Such results help provide an explanation for the differences in severity of corneal manifestation found in *Lum*$^{-/-}$ and *Kera*$^{-/-}$ mice. The results also suggest a critical level of small proteoglycans to be essential for collagen organization but that overabundance is not detrimental to extracellular matrix morphogenesis.

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Lumican is a member of the small leucine-rich proteoglycan (SLRP)[1] family with expression reported in cornea, sclera, aorta, cartilage, liver, skeletal muscle, kidney, pancreas, brain, placenta, and lung (1–6). Lumican co-localizes with collagen fibrils in the corneal stroma and has been hypothesized to be involved in modulation of the highly organized collagen matrix required for corneal transparency (7,8). These predictions were substantiated by findings of corneal opacity, skin fragility, and abnormally large collagen fibril diameters and disorganized interfibrillar spacing present in lumican-null mice (1,7,9–11). More recently, studies have showed lumican involvement in cell migration and proliferation during embryonic development and wound healing (12–15). The delayed epithelial wound healing phenotype in *Lum*$^{-/-}$ mice is potentially due to the involvement of lumican in cellular migration, adhesion, and/or proliferation (12,16). Under normal conditions, lumican is not expressed by epithelial cells, but transient expression is reported by migrating cells during wound healing and development (12,17). Impairment of cell migration and proliferation may partially explain the delay of epithelial wound healing in lumican-null mice. Recent reports have also showed delayed epithelial-mesenchymal transition in lumican-null mice. Lumican-null mouse lens epithelial cells showed decreased α-smooth muscle actin expression and the delayed epithelial-mesenchymal transition induction by transforming growth factor β-2 *in vitro* (18). A role for lumican has also been suggested in growth and metastasis of breast, colon, and pancreatic cancer (19–21) and in cellular apoptosis (21). A cell surface receptor for lumican has also been demonstrated (22). These observations demonstrate lumican to have multiple functions, and it may serve as a matrikine in regulating cellular activities via interaction with integrin and/or growth factor receptors besides serving as a component of ECM, similar to what has been suggested for many other ECM components (23–25). The full extent of cellular functions mediated by lumican, however, remains to be determined.

Keratan sulfate-containing proteoglycans (KSPGs) are uniquely abundant in the cornea and have long been thought to be essential for corneal transparency. Lumican constitutes only about half of corneal KSPG. Most of the remaining corneal keratan sulfate modifies keratocan, a protein with high sequence similarity to lumican. In adult tissues, keratocan is limited to corneal stroma, and keratocan expression is considered a phenotypic marker for keratocytes. Lumican-null (*Lum*$^{-/-}$) mice manifest corneal opacity, skin fragility, and impaired collagen fibrillogenesis (1,12); however, ablation of the keratocan gene (*Kera*) only results in a subtle manifestation of thin but transparent cornea in which little changes of the collagen matrix can be detected (26,27). Because of the high degree of similarity in amino acid sequence, post-translational modification, corneal localization, and apparent function, there has been no immediately apparent explanation for the different outcomes resulting from the ablation of *Lum* and *Kera* genes.

In the current study, we have approached the question as to the function of the KSPG in the cornea by overexpressing lumican in the corneal stroma of the mouse. This experiment explores the potential of a stoichiometric relationship between corneal collagen KSPG. The results indicate that the presence of excess lumican does not have any adverse effects on corneal morphogenesis, collagen organization, or corneal transparency. However, using a number of different experimental approaches, lumican expression was found to exert a direct effect on the expression of keratocan. These findings provide a new explanation for the differences of clinical manifestations in corneas of *Lum*$^{-/-}$ and *Kera*$^{-/-}$ mice and document a novel cellular regulatory function of lumican.

[1]The abbreviations used are: SLRP, small leucine-rich proteoglycan; ECM, extracellular matrix; KSPG, keratan sulfate-containing proteoglycan; siRNA, small interfering RNA; RT, reverse transcription; CMTF, confocal microscopy through focusing.

EXPERIMENTAL PROCEDURES

Preparation of Kera-Lum Transgenic Mice

Two oligonucleotides, a 5′ ClaI (CCA TCG ATG CCA CCA TGG AGA CAG AC) primer and a 3′ SalI (ACG CGT CGA CCA GAT CCT CTT CTG AGA TG) primer, were synthesized by the University of Cincinnati DNA core facility (Cincinnati, OH) and used to generate lumican cDNA using 100 ng of pSecLum as a template in a standard PCR (28). The PCR product was digested by ClaI and SalI, gel-purified, and ligated to the keratocan 3.2-kb promoter in the pBSK vector (29). The fidelity of the *Kera-Lum* minigene was verified by nucleotide sequence in both strands. A schematic of this construct is shown in Fig. 1*A*. The *Kera-Lum* minigene was released by FspI and SacII digestion and used for pronuclei injection of fertilized mouse eggs by the Cincinnati Children's Hospital Research Foundation Transgenic Core Facility (Cincinnati, OH). To identify transgenic mice, tail DNA from mice was used for genotype analysis by PCR with a forward Ktn9 (5′-CCT AAC ACC AGC CAC AGG ACT) and a reverse BGH (5′-TAG AAG GCA CAG TCG AGG) primer pair using the following PCR cycle: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 35 cycles, and 72 °C for 10 min.

RT-PCR

RNA was extracted from transgenic mouse cornea using TRI-reagent™ (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendations. Reagents used in this assay, unless otherwise specified, were from Promega (Madison, WI). cDNA was synthesized in a 200-μl reaction mixture containing 40 μl of 5× reverse transcription buffer, 20 μl of 0.1 M dithiothreitol, 8 μl of 25 mM dNTPs, 10 μl of Rnasin (40 units/μl), 10 μl of random hexamers (50 μM) (Amersham Biosciences), 10 μl of avian myeloblastosis virus reverse transcriptase (9.5 units/μl), and 10 μg of heat-denatured corneal total RNA extracts from transgenic mouse corneas. Diethylpyrocarbonate-treated water was added to bring the final reaction volume to 200 μl. The reaction was then incubated sequentially at 22 °C for 10 min, 42 °C for 90 min, and 100 °C for 2 min and then immediately placed on ice. A 20-μl aliquot of the described reverse transcription reactions was added to 80 μl of a PCR mixture containing 8 μl of 10× PCR buffer (no $MgCl_2$), 8 μl of 25 mM $MgCl_2$, 10 μl of 20 ng/μl primers (Ktn9, 5′-CCT AAC ACC AGC CAC AGG ACT; BGH, 5′TAG AAG GCA CAG TCG AGG), 0.5 μl of *Taq* polymerase (5 units/μl), and 45.5 μl of double-distilled H_2O. The PCR was performed for 35 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min for 35 cycles, followed by a 5-min extension time at 72 °C at the end of 35 cycles.

Western Blot

Mice were sacrificed, and corneas were immediately excised using scissors and forceps and placed in 1 ml of a solution containing 4 M guanidine HCl, 0.01 M sodium acetate, 0.01 M Na-EDTA, 0.005 M benzamidine-HCl, and 0.1 M ϵ-amino-*n*-caproic acid and allowed to incubate at 4 °C overnight. Corneas were then homogenized using a Tissuemizer® (Tekmar, Cincinnati, OH) three times for 30 s each time. After homogenization, samples were incubated at 4 °C overnight. Following this incubation, the samples were centrifuged, and the supernatant was dialyzed against double-distilled H_2O overnight at 4 °C in a dialysis bag. Precipitates were collected by centrifugation, air-dried for 10 min, and resuspended in 100 μl of 6 M urea in 0.1 M Tris acetate, pH 7.4. The samples were then centrifuged, supernatants were collected, and optical densities were measured with a spectrophotometer at 280 nm. Routinely, about 7–10 μg of total protein could be extracted from each cornea. Samples were then digested with 0.1 unit/ml endo-β-galactosidase (Sigma) at 37 °C overnight. Western blots were performed as described previously (30) and developed using an ECL development kit, ECL-Plus® (Amersham Biosciences) according to the manufacturer's recommendations. Band intensities

were measured using NIH Image (National Institutes of Health, Bethesda, MD). Antibodies to mouse lumican and mouse keratocan were used as primary antibodies (12,26).

In Vivo Confocal Microscopy

In vivo confocal microscopy through focusing (CMTF) was used to measure corneal epithelial thickness, stromal thickness, and corneal haze in the nontransgenic, *Lum38*, and *Lum5* transgenic mice (three animals for each group). Mice were initially anesthetized by intraperitoneal injection of ketamine HCl (100 mg/kg body weight, Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg body weight, Akorn, Inc., Decatur, IL). Eyelids were then held open using tape, and the central cornea was scanned using a tandem scanning confocal microscope (Tandem Scanning Corp., Reston, VA) equipped with a ×24 surface contact objective (numerical aperture = 0.6, working distance = 1.5 mm). Thickness and light scattering measurements were obtained using previously described techniques (31–33). During observations, both eyes were kept moist using topically applied artificial tear solution (Celluvisc, Allergan Inc., Irvine, CA) to avoid corneal desiccation, and a drop of artificial tears was placed on the tip of the objective to serve as an immersion fluid. CMTF collects a series of digital, two-dimensional images through the cornea at known intervals that can later be reconstructed to generate a three-dimensional image from which quantitative measurements of corneal sublayer thickness and light scattering can be obtained. Three CMTF scans in the forward, anterior to posterior, direction were obtained from the right eye of each animal. Depth intensity profiles were then generated, and thickness measurements for the epithelium and stroma were then obtained using previously published formulas (32,33). To assess light scattering, the area under the pixel intensity curve (U_{auc}) corresponding to the corneal stroma was integrated to estimate of backscattering light using previously published techniques (33, 34). The average epithelial thickness, stromal thickness, and corneal haze were calculated from three separate *z*-scans from the same eye, and then the mean and S.D. from three separate animals were calculated.

Transmission Electron Microscopy

Corneas from three mice per line were analyzed by transmission electron microscopy. Briefly, the corneas were fixed *in situ* in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM $CaCl_2$ for 2 h (8). The corneas were then postfixed with 1% osmium tetraoxide and *en bloc* stained with uranyl acetate, 50% ethanol. After dehydration in an ethanol series, followed by propylene oxide, the corneas were infiltrated and embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenyl succinic anhydride, and DMP-30 (Electron Microscopy Sciences). Thin sections were cut using a Reichert UCT ultramicrotome and a diamond knife and stained with 2% aqueous uranyl acetate, 1% phosphotungstic acid, pH 3.2. Sections taken from the central cornea and the anterior and posterior stroma were analyzed independently using electron microscopy. Sections were examined and photographed at 75 kV using a Hitachi 7000 transmission electron microscope.

Fibril diameter analyses were done for the nontransgenic and transgenic lines. For each line, fibril diameters were measured in micrographs from nonoverlapping regions of the anterior and posterior stroma from the central taken at ×31,680. Micrographs were randomly chosen in a masked manner from the different groups and digitized, and diameters were measured using a RM Biometrics-Bioquant Image Analysis System (Memphis, TN).

Northern Hybridization

Total RNA was extracted from two mouse corneas using TRI-reagent™ (Molecular Research Center, Cincinnati, OH). Ten μg of total RNA were subjected to Northern blot hybridization with ^{32}P-labeled mouse keratocan cDNA probe as described previously (35). Hybridization signals were detected with a PhosphorImager (Amersham Biosciences).

Intrastromal Injection

Lumican-null mice were utilized in this study after preoperative examination for exclusion criteria such as ocular disease, wound, or infection (12). Animal care and use conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Mice were anesthetized by intraperitoneal injections of ketamine (100 μg/g body weight) and xylazine (13 μg/g body weight). One drop of Alcaine®, 0.5% proparacaine hydrochloride ophthalmic solution (Alconlabs, Fort Worth, TX) was applied to each eye prior to surgery as a topical anesthetic. The mice were subject to intrastromal injection (36) and observed during a recovery period until awakening in a heated chamber (37). A small incision was first made in the corneal epithelium using a 30-gauge hypodermic needle. A 33-gauge needle attached to a 25-μl syringe was passed through the incision into the stroma. The needle was pushed several μm into the stroma, and up to 5 μg of pSecLum (28) plasmid DNA (2 μg/μl) was injected into corneal stroma under a stereomicroscope.

Immunohistochemistry

Eyes were enucleated 5 days after intrastromal injection and subjected to hematoxylin and eosin staining or immunostained for lumican and keratocan. Keratocan and lumican immunostaining was performed in the following manner. Five-μm paraffin sections were deparaffinized, blocked, and processed for indirect immunostaining using epitope-specific rabbit antibodies against synthetic mouse lumican (8 μg/ml) and mouse keratocan (40 ng/ml) peptide as primary antibodies, and peroxidase-conjugated goat anti rabbit-IgG antibodies were used as secondary antibodies (Southern Biotechnologies, Birmingham, AL) as previously reported (12,26). Antibody reaction was visualized with 3,3′-diaminobenzidine. The specimens were counterstained with hematoxylin, dehydrated through a graded ethanol series, mounted in balsam, and observed by light microscopy.

Lumican Knockdown

Cultures of primary bovine keratocytes were plated at 4×10^4 cells/cm^2 for 48 h in serum-free medium as previously described (38). These were treated with Lipofectamine 2000 (Invitrogen) 4 μl/35-mm dish either alone or complexed with a mixture of 421-bp RNA duplexes against bovine lumican (Dharmacon; Smartpool) at a final concentration of 100 nM in 1% platelet-poor horse serum. The siRNA sequence was derived from the open reading frame of bovine lumican (GenBank™ accession NM_173934) using a Dharmacon algorithm. The location and sequence of the duplexes were as follows: 223, GAAUGUAACUGCCCUGAAAUU; 1100, CGAAUGAGAUCACUGUUAAUU; 759,CAACAUCCCUGACGAGUAUUU; 146, GCACCUAUCCUGAUUACUAUU. 72 h after transfection, the medium was changed, and 24 h later, cells were lysed in 4 M guanidine HCl, 20 mM Tris, pH 7.4, and proteoglycans were recovered by centrifugation after dialysis against water, as described above. Proteoglycans were recovered from the medium by ion exchange chromatography, dialysis, and lyophilization (39). Lyophilized samples were dissolved in 6 M urea 0.1 M Tris acetate, pH 6, and digested overnight at 37 °C with 10 milliunits/ml endo-β-galactosidase. Lumican and keratocan in the digests were detected by Western blotting after separation on a 4–20% SDS-polyacrylamide gel using a monoclonal antibody to bovine lumican (provided by Bruce Caterson). Total RNA was purified from similar cultures at 72 h for real time quantitative PCR of keratocan, aldehyde dehydrogenase 3A1, and biglycan as previously described (39).

Keratocan Promoter Assay

Intrastromal injection of plasmid DNA constructs was performed in lumican-null 12-week-old mice as previously described (36). 2 μg of pKera3.2INT-β-GEO was co-injected with either 2 μg of pSecLumWT or pSecTag2A empty vector in a 2-μl volume (28). Four days following instrastromal injection, corneas were excised (n = 4), and reporter gene activity was measured. β-GEO reporter analysis was performed using a β-galactosidase enzyme assay kit (Promega, Madison WI) according to the manufacturer's protocol. In brief, corneal total extracts were isolated by homogenization with a Polytron® homogenizer (Brinkmann Instruments, Westbury, NY) in 0.5 ml of reporter lysis buffer included in the kit. Protein concentrations were determined using a BCA protein assay reagent kit (Pierce), according to the manufacturer's recommendations. 50 μg of total corneal protein was used to determine the β-galactosidase activity and plotted on a standard curve to determine β-galactosidase activity/cornea. A t test was used to determine statistical significance (α = 0.05) between control and experimental groups.

RESULTS

The initial goal of this study was to investigate the role of lumican in the corneal stroma by generating transgenic mice with tissue-specific lumican overexpression. To this end, a lumican minigene encoding a lumican-c-Myc tag fusion protein under the control of the 3.2-kb keratocan promoter was prepared (29). Fig. 1*A* shows a diagram of the Kera-Lum minigene used to create transgenic mice by pronuclei injection. Fig. 1*B* shows PCR genotyping with a nontransgenic (*NTG*) and positive control (+), and the transgenic mouse lines *Kera-Lum5*, *-25*, and *-38* used for this study were positive for the 1500-bp reporter gene DNA fragment. The transcript expression was then analyzed by RT-PCR. *Kera-Lum5*, *-25*, and *-38* transgenic mouse lines were positive for a 1016-bp cDNA of Lum mRNA derived from the transgene using the same Ktn9 and BGH primers used for genotyping and were further analyzed in this study (Fig. 1*C*). Three other founder lines, *Kera-Lum50*,*-55*, and *-58*, were also positive for the transgene (data not shown) by PCR but negative for the presence of mRNA of the transgene by RT-PCR; these animals were not analyzed further (data not shown). To confirm transgene translation, Western blot analysis was performed on transgenic and nontransgenic mice by extracting the KSPGs from corneas, loading 7.5 μg of protein in each well, and probing with anti-lumican (Fig. 1*D*) (12). Lumican in corneas of the *Kera-Lum* transgenic lines was 3–4-fold higher than that of nontransgenic corneas determined using NIH Image software (National Institutes of Health, Bethesda, MD). Further confirmation of transgene expression was performed by utilizing the c-Myc epitope tag present in the carboxyl terminus of the recombinant protein (Fig. 1*D*). Western blot analysis was performed on 1.0 μg of extracted protein, and anti-c-Myc antibody (Research Diagnostics Inc., Flanders, NJ) was used. Specimens from transgenic mice showed a 45-kDa protein recognized by anti-c-Myc antibody, but no signal was detected in nontransgenic corneas, further confirming the presence of recombinant lumican encoded by the transgene in the cornea.

The data summarized in Table I demonstrate that there were no significant differences between the nontransgenic and transgenic mice for epithelial thickness, stromal thickness, and haze. The average epithelial and stromal thicknesses in the nontransgenic mice were 48.42 ± 3.29 and 52.33 ± 5.46 μm, respectively. Light scattering in the nontransgenic mice was 565 ± 90.14 U_{auc}. Although the level of light scattering appeared higher in the transgenic mice compared with the nontransgenic, the increased scattering was not significantly different between nontransgenic and transgenic *Kera-Lum* mice. Nevertheless, transgenic mice showed slightly elevated levels of corneal haze compared with the nontransgenic mice. Furthermore, animals showing higher levels of expression of the transgene (*Kera-Lum38*) show higher levels of haze than transgenic mouse lines with lower levels of expression (*Kera-Lum5*).

Electron microscopy examination revealed that the stromal architecture was nearly identical among nontransgenic and the two transgenic mouse lines (*Kera-Lum5* and -*38*; Fig. 2). Fibril packing and spacing were comparable in normal and overexpressing stromas. No significant differences were observed between the anterior and posterior stroma. Occasionally in the transgenic lines there appeared to be limited regions with less ordered packing. Fibril diameters were not significantly different in nontransgenic *versus* transgenic lines. Fibril diameters from the anterior stroma were 24 ± 4nm (n = 406) and 23 ± 3 nm (n = 484), whereas values for the posterior stroma were 22 ± 3 nm (n = 373) and 24 ± 3 nm (n = 462) for nontransgenics and transgenic, respectively. The fibril diameters were in the 9–36-nm range for all samples.

After characterizing lumican expression and corneal architecture of the transgenic founder lines, keratocan expression was analyzed to determine whether lumican overexpression might impact corneal KSPG equilibrium. Keratocan expression was determined through Western blot analysis using an anti-keratocan antibody (26). Surprisingly, Fig. 3*A* shows 5–6-fold higher keratocan expression in cornea of transgenic mice than that of nontransgenic littermates, suggesting an up-regulated keratocan expression caused by excess lumican. To further examine whether lumican may modulate keratocan expression, proteoglycans were extracted from corneas of $Lum^{+/+}$, $Lum^{+/-}$, and $Lum^{-/-}$ knockout littermates and nontransgenic and *Kera-Lum* transgenic mice. Fig. 3*B* shows the results of a Western blot in which proteins from corneal extracts were separated by SDS-PAGE. Keratocan expression was slightly decreased in the heterozygous $Lum^{+/-}$ mice compared with $Lum^{+/+}$ wild type mice, but a marked decrease of keratocan expression was detected in the homozygous $Lum^{-/-}$ animals. To further confirm the impact of lumican levels on keratocan expression, Northern hybridization probing for keratocan mRNA was performed with total corneal RNA extracted from $Lum^{-/-}$, $Lum^{+/-}$, wild-type $Lum^{+/+}$ littermates, and nontransgenic and *Kera-Lum* transgenic mice (Fig. 3*C*). Keratocan mRNA levels paralleled those of the Western blot analysis, demonstrating that the keratocan mRNA of $Lum^{-/-}$ mice was decreased compared with that of heterozygous $Lum^{+/-}$ and wild-type $Lum^{+/+}$ mice. Moreover, the *Kera-Lum* transgenic mouse showed an increase in keratocan mRNA as compared with the nontransgenic mouse. These results substantiate the hypothesis that lumican regulates keratocan expression by the stromal keratocytes.

The results of these analyses prompted further investigation to confirm this novel role of lumican in the keratocyte. Intrastromal naked DNA injection was previously shown to drive expression of a reporter gene in the corneal stroma (37). To further verify the role of lumican in keratocan expression, pSecLum plasmid cDNA (5 μg) was injected into the corneal stroma of lumican-null mice to determine whether an increase in lumican could stimulate an increase in keratocan expression, pSec-empty vector was used in control experiments. Five days postinjection, mice were sacrificed, eyes were enucleated, and histology was performed. Fig. 4 shows empty vector and pSecLum plasmid DNA-injected eyes stained with hematoxylin and eosin and immunostained for lumican. Empty vector-injected corneas exhibit the absence of lumican expression. Lumican was detected in pSecLum-injected corneas. To confirm the increased *Kera* expression induced by Lum, Western blot analysis was performed to determine keratocan in intrastromal pSecLum-injected corneas of the $Lum^{-/-}$ mouse. As shown in Fig. 5, *lower panel*, delivery of pSecLum plasmid DNA to the corneal stroma results in lumican expression detected by anti-lumican antibodies; no lumican could be detected using the control empty vector at 5 days post-injection. Fig. 5, *upper panel*, also shows a 2-fold increase in keratocan expression by Western analysis after injection of pSecLum plasmid DNA as compared with the empty vector control using the NIH Image program. This result further supports the notion that lumican can regulate keratocan expression.

In order to corroborate the *in vivo* analysis, keratocan expression was examined in primary keratocyte cultures *in vitro* in which lumican has been reduced by siRNA transfection (Fig. 6). Fig. 6*A* shows that in a mock-transfected culture lumican was recovered primarily from the

proteoglycan fraction secreted into the medium during a 24-h period (*lane 2*). After a 48-h transfection with lumican-specific siRNA, lumican present in the medium was suppressed by over 90% (*lane 4*). Keratocan and aldehyde dehydrogenase 3A1 mRNA pools showed a marked down-regulation of both of these keratocyte-specific genes after lumican knockdown (Fig. 6*B*). Biglycan mRNA, on the other hand, was not altered in siRNA-treated cells.

Upon confirming the ability of lumican to regulate keratocan expression using *in vivo* and *in vitro* models, the mechanism by which this occurs was investigated. To further confirm that lumican can indeed regulate keratocan expression, a keratocan promoter driven reporter gene (pKera3.2INT-β-GEO) was co-injected intrastromally into $Lum^{-/-}$ mice with either a lumican-expressing minigene (pSecLumWT) or an empty vector control (pSecTag2A). 4 days following intrastromal injection, corneas were harvested, and β-galactosidase activities were determined. Fig. 7 shows a histogram of the β-galactosidase units/cornea ($n = 4$) of the vector or lumican minigene co-injected with pKera3.2INT-βGEO. Lumican expression significantly increases the activity of the keratocan promoter, demonstrating the ability of lumican to regulate keratocan gene expression at the promoter level.

DISCUSSION

Keratocan and lumican are regulators of collagen matrix organization and assembly in the corneal stroma (40). Besides regulating collagen fibrillogenesis, lumican plays a role in several biological processes such as wound healing, epithelial-mesenchymal transition, and tumorigenesis (12,18,19,21,21,41). The results reported here indicate a novel biological role of lumican as a modulator of keratocan gene expression by keratocytes.

The characterization of the lumican and keratocan-null mouse were previously reported (1, 12,26). Whereas the lumican-null mouse has a profound phenotype, including corneal opacity and skin fragility due to altered collagen fibrillogenesis, delayed corneal epithelium wound healing (1,12), and delayed epithelial-mesenchymal transition of injured lens (18), the keratocan-null mouse exhibited subtle clinical manifestation of thin but transparent cornea and did not develop corneal opacity as examined out to 12 months (26,27). Further examination of the keratocan-null mouse showed no alteration of other SLRPs in the corneal stroma, including lumican (26). The result shown in Fig. 3 depicts the decrease in keratocan expression in the $Lum^{+/-}$ and a further decrease in the $Lum^{-/-}$ mice as compared with wild type $Lum^{+/+}$ littermates. This suggests that the corneal phenotypes (opacity, perturbed fibrillogenesis, and thin corneal stroma) in the lumican knockout animals are not solely the result of an absence of lumican expression but also the result of a significant decrease of keratocan expression by corneal stroma of $Lum^{-/-}$ mice. It is of interest to note that there was a marked increase of lumican expression in tendon of fibromodulin null mice ($Fmod^{-/-}$). It was suggested that the up-regulation of lumican might in part rescue the tendon phenotype of $Fmod^{-/-}$ mice (42). The observations implicate that the expression of members of the SLRP family may be regulated by other members in the family. The results of our studies provide direct evidence to substantiate such a hypothesis.

The initial goal of this study was to better define the role of lumican in the cornea using transgenic animals overexpressing lumican on a wild type background. If collagen fibrillogenesis requires KSPGs and collagen in a strict stoichiometric ratio, then such an overexpression would be expected to alter corneal morphogenesis, fibril diameter, and transparency of the transgenics. The absence of such corneal pathologies indicates that overabundance of KSPG proteins is not detrimental to correct corneal morphogenesis. The keratocan knockouts and heterozygous $Lum^{+/-}$ mice both have normal stromal collagen matrix, and only the $Lum^{-/-}$ mice show markedly altered stromal collagen. Our current data show that the lumican knockouts not only lack lumican but also have reduced keratocan. Based on these

findings, we propose that correct corneal morphogenesis requires a minimal concentration of KSPG proteins and that a strict ratio is not necessary. Excess KSPG appear not to be detrimental. This may be in part explained by the presence of a limited number of KSPG binding sites on collagen molecules (43).

Also shown was a decrease in lumican expression resulted in decreased keratocan expression. Furthermore, intrastromal injection of lumican cDNA into lumican-null animals resulted in lumican expression, but more importantly an increase in keratocan expression. Taking this finding into account coupled with the unaltered lumican expression in the keratocan knockout mouse, lumican is capable of regulating keratocan expression in the adult cornea. The idea that lumican may serve as a regulatory molecule for keratocan expression was explored to determine the mechanism by which lumican can regulate keratocan expression using an *in vivo* promoter assay where the presence of lumican was able to significantly increase keratocan promoter activity in $Lum^{-/-}$ mice as shown in Fig. 7.

Our observations are consistent with the suggestions that members of SLRP family may have additional functions besides serving as components of ECM (44). For example, decorin is another SLRP family member that shares similar functions with lumican. Not only is decorin an important molecule in ECM stabilization; it has been shown to be involved in cell proliferation and migration and protein synthesis. Analogous to the phenotypes found in lumican-null mice, decorin-null mice also exhibit skin fragility and impaired collagen fibrillogenesis (45). Furthermore, lumican and decorin are both maximally expressed in quiescent cells. Several studies have suggested the ability of ECM proteins to function as low affinity ligands for growth factor receptors and coined the term “matrikines” (23). Decorin has been identified as a matrikine, and its ability to initiate signaling pathways such as the ERK1/ERK2 pathways (46,47) and receptor tyrosine kinases provides a signaling link between the ECM and nuclear function (48–51).

A previous report suggests the ability of lumican to serve as a ligand for macrophage receptor (s) in its low sulfated form (22). We have recently observed that stromal cells and polymorphonuclear neutrophils bind lumican.[2] These observations are consistent with the existence of cell surface receptor(s) of lumican. The actual receptor responsible for this has not been identified to date, but this receptor may also exist on the keratocyte. It is plausible to hypothesize that lumican modulates the expression of keratocan and/or other genes by keratocytes. A receptor for lumican and/or KSPGs would logically be present on the keratocyte for maintaining extracellular matrix equilibrium in the corneal stroma, since collagen fibrillogenesis in the cornea proceeds in such a highly organized fashion to form a transparent tissue that regulation of the factors involved is paramount.

The implication that lumican involvement in tumorigenesis is substantiated by the observation in which expression of lumican has been associated with colorectal epithelial cells with mild reactive dysplasia (42) and breast carcinomas (19,41,52). However, the precise role of lumican involvement in tumorigenesis remains elusive, since conflicting data of the levels of lumican expression during tumorigenesis has been reported in various tumors. For example, high lumican expression levels were detected in invasive carcinomas (19), whereas low lumican expression correlates with large tumor size (52). The presence of lumican receptor and multiple functions of lumican may provide an explanation for the observed variations of lumican in tumorigenesis. The pathways and mechanisms governing these processes are unclear, but the combination of the expression of lumican and its receptor(s) may contribute to the outcomes of cell behaviors during tumorigenesis.

[2]E. C. Carlson, C.-Y. Liu, T. Chikama, Y. Hayashi, C. W.-C. Kao, J. L. Funderburgh, and W. W.-Y. Kao, unpublished data.

Taken together, our results strongly support the hypothesis that lumican is a regulatory protein for keratocan expression at the promoter level in the adult mouse corneal stroma. Nevertheless, the signaling mechanism behind this novel function of lumican has yet to be determined. Like decorin, another SLRP family member, lumican may function as a matrikine through a receptor yet to be identified. There are many implications of lumican functioning as a ligand and initiating intracellular signaling pathways that have the ability to regulate expression of various genes.

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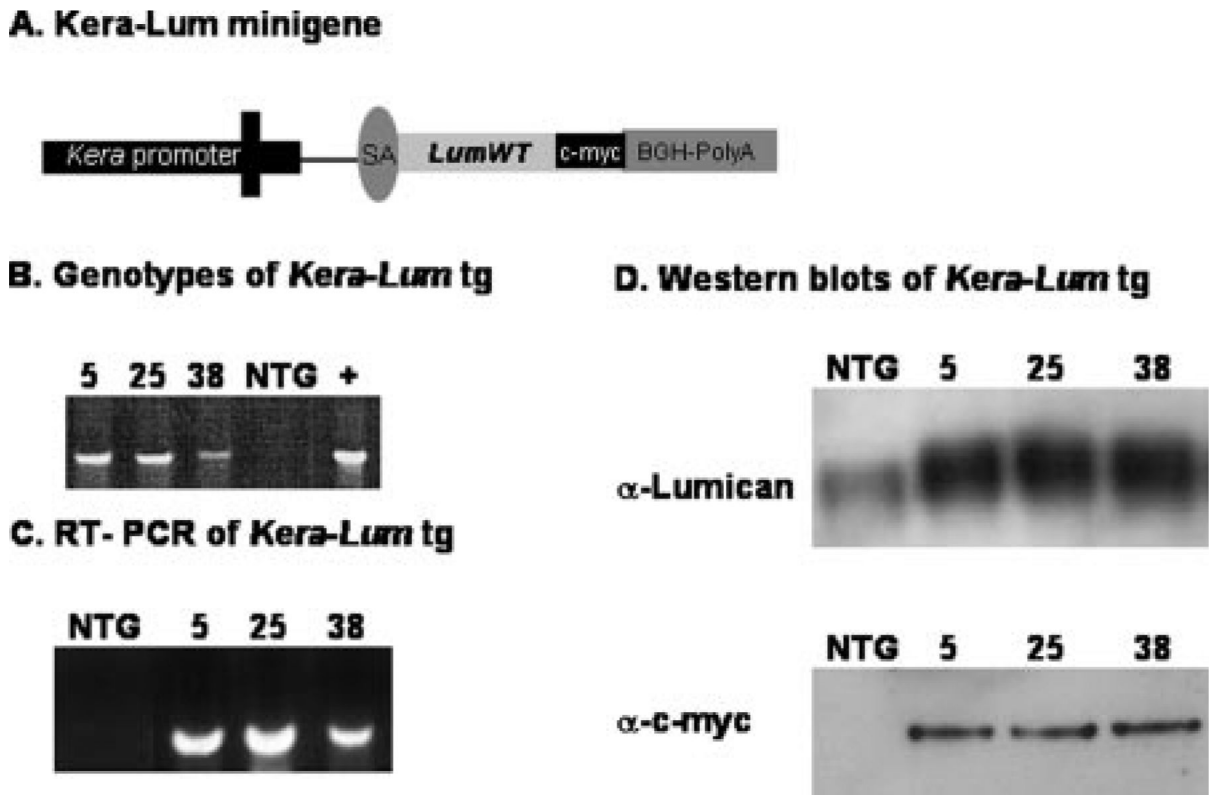

Fig. 1. Schematic of *Kera-Lum* minigene used for transgenic mouse generation

Lum cDNA containing the c-Myc tag was ligated to the 3.2-kb keratocan promoter followed by a BGH polyadenylation signal with pBSK vector (*A*). Shown is PCR genotyping of three lines of *Kera-Lum* transgenic mice, a nontransgenic littermate (*NTG*), and a positive plasmid DNA control. The presence of the 1,500-bp fragment is positive for the transgenic construct (*B*). Shown is RT-PCR of *Kera-Lum5*, *-25*, and *-38* of the *Kera-Lum* transgenic mice and a nontransgenic control. The presence of the 1,016-bp DNA fragment is positive for the transcript from the transgene (*C*). Shown are Western blots using anti-lumican and c-Myc antibodies from partially purified corneal extracts from three lines of transgenic animals compared with a nontransgenic control probing for lumican. The transgenic animals show a 3–4-fold increase in lumican (about 45 kDa) expression as compared with the nontransgenic control. c-Myc Western blotting revealed a positive signal in all three transgenic lines but no signal in the nontransgenic mice (*D*).

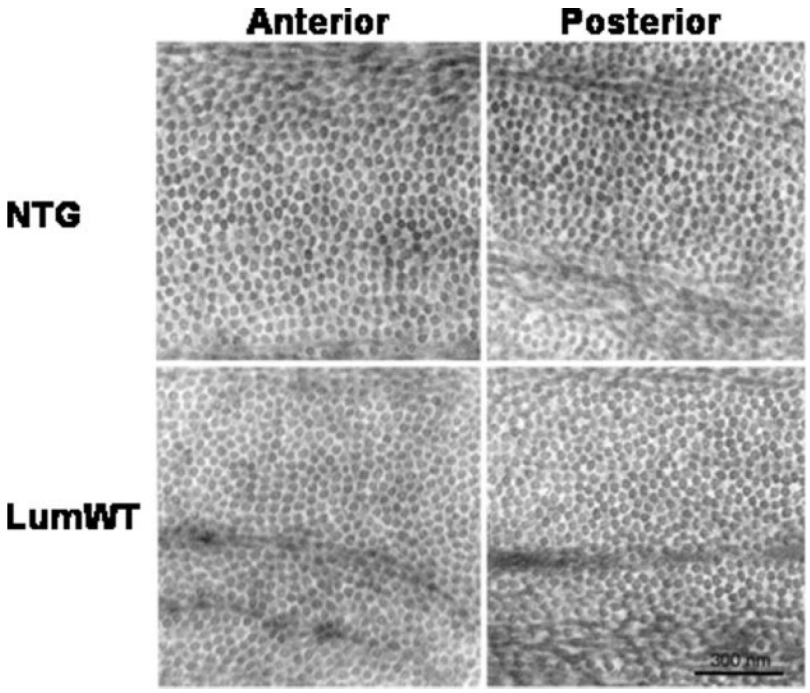

Fig. 2. Transmission electron micrographs showing similar stromal architecture in stromas from nontransgenic and transgenic mouse corneas

The collagen fibril diameters in corneas of wild type and transgenic mice are virtually identical. In addition, fibril packing and spacing are comparable in normal and overexpressing lines. Identical results were observed in both the anterior and posterior regions of the stroma. *Bar*, 300 nm.

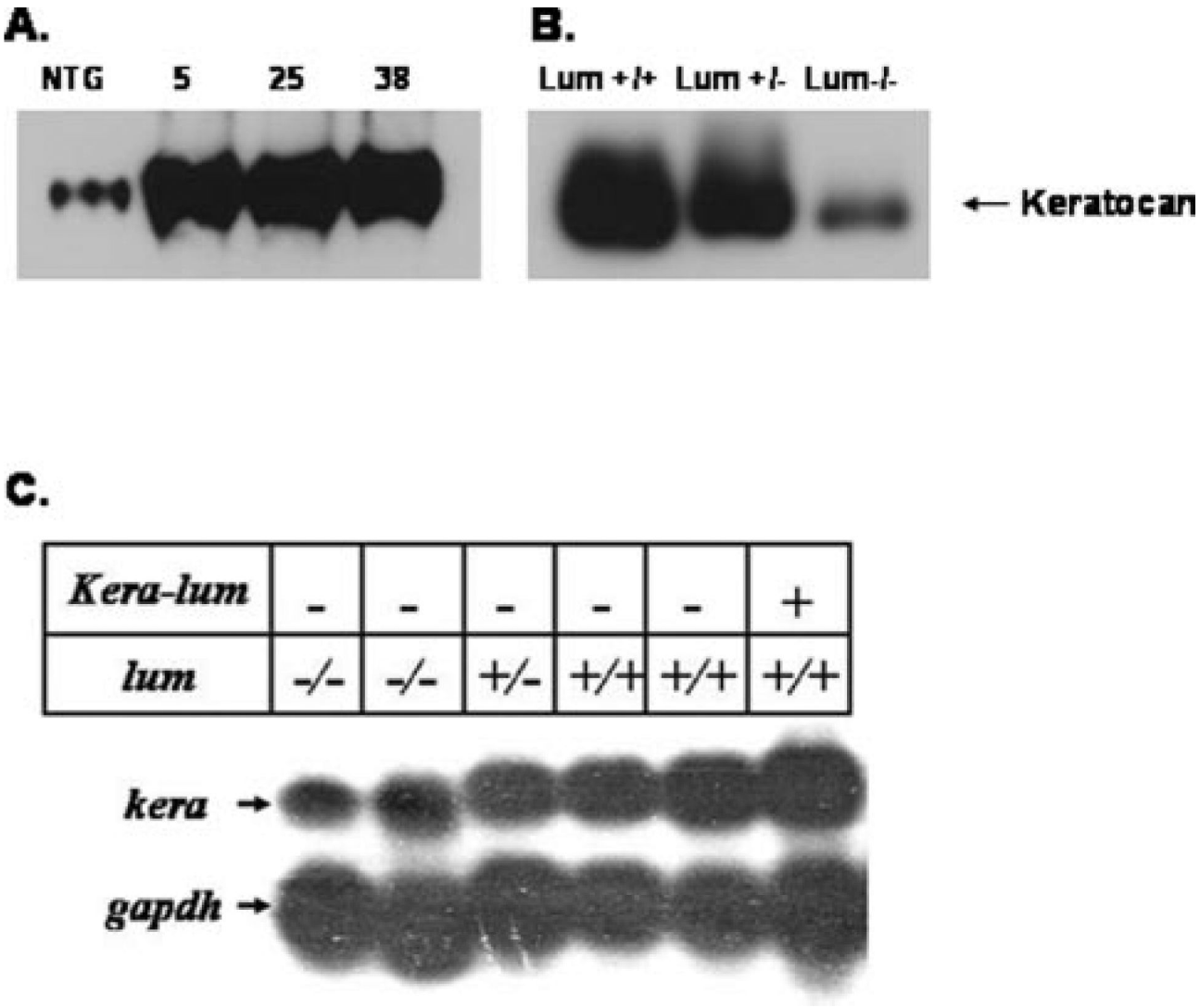

Fig. 3.

Western blots probing for keratocan in the three transgenic lines (*A*) and in $Lum^{+/+}$, $Lum^{+/-}$, and $Lum^{-/-}$ mouse corneas (*B*). Keratocan is up-regulated ~5-fold in the *Kera-Lum* lines as compared with the nontransgenic control. Keratocan levels decrease in the heterozygous ($Lum^{+/-}$) and homozygous ($Lum^{-/-}$) knockout corneas. Northern blotting hybridization of total RNA (10 μg) from two corneas of $Lum^{-/-}$, $Lum^{+/-}$, wild-type $Lum^{+/+}$, nontransgenic littermate ($Lum^{+/+}$), and *Kera-Lum* transgenic mice. Keratocan mRNA is down-regulated in $Lum^{-/-}$ mouse corneas and up-regulated in the *Kera-Lum* transgenic mouse (*C*).

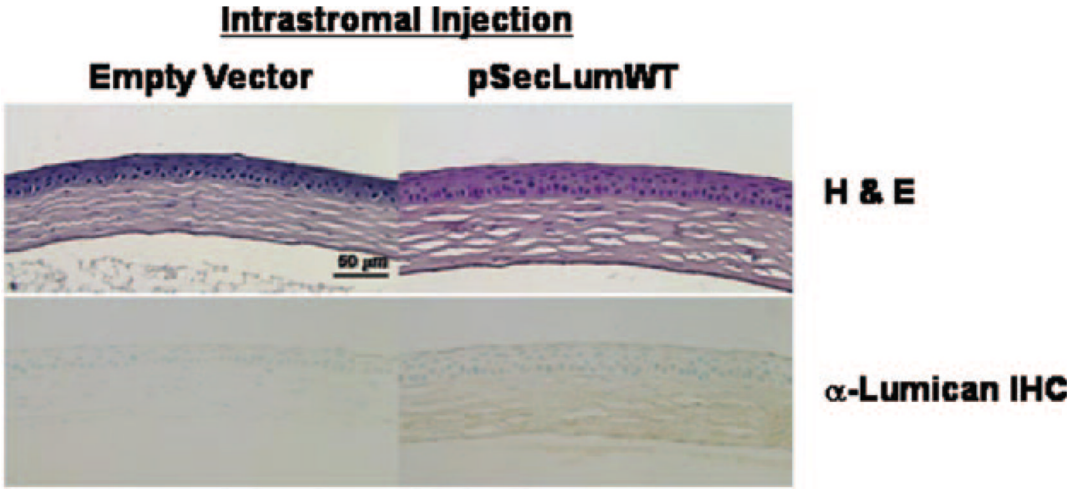

Fig. 4. Histological analyses of lumican-null mouse corneas 5 days after intrastromal injection of pSecLum or empty vector control plasmid DNA

Hematoxylin and eosin (*H&E*) staining revealed no significant morphological changes between empty vector- and pSecLum-injected corneas. Immunohistochemistry using anti-lumican antibody detects the presence of lumican in the pSecLum-injected cornea.

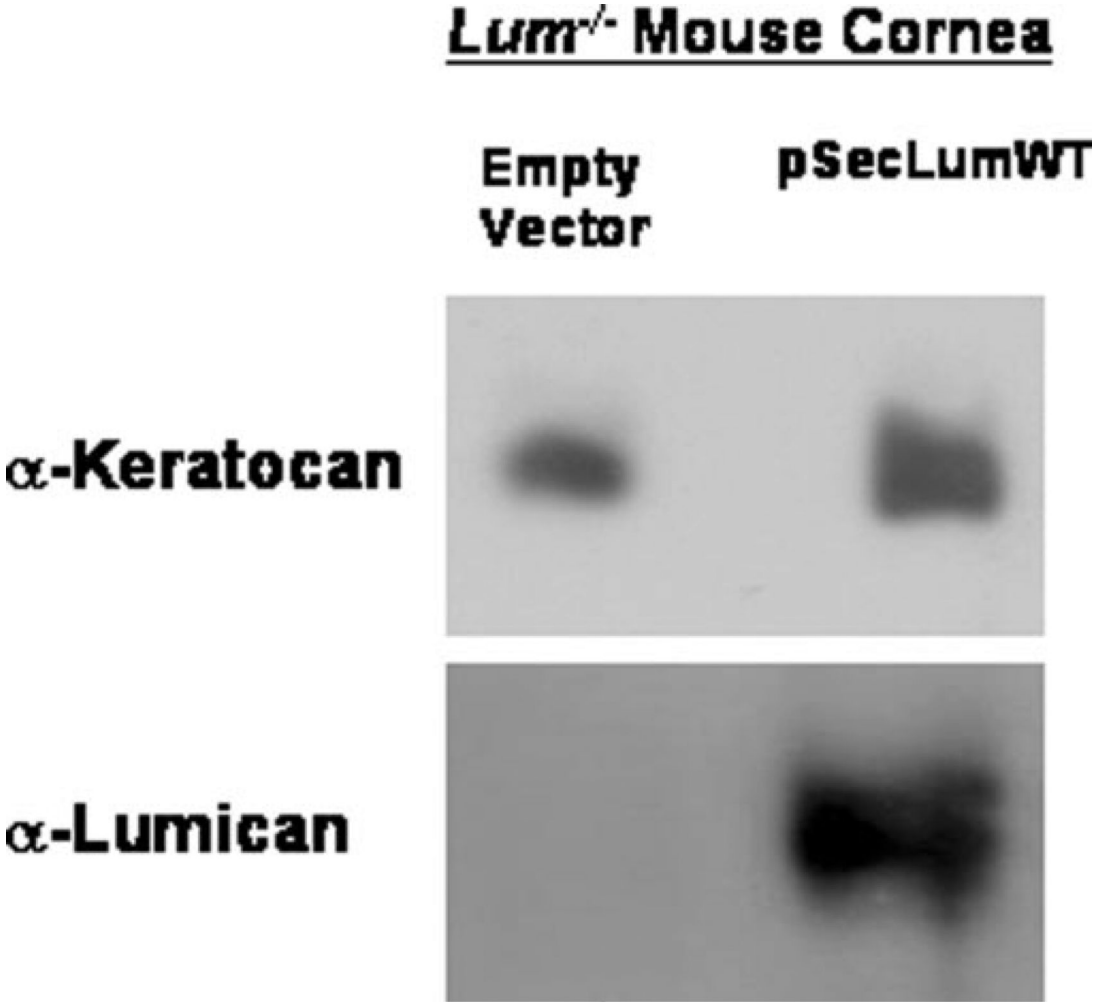

Fig. 5. Western blot for lumican (*bottom*) and keratocan (*top*) of corneal extracts from *Lum*$^{-/-}$ mice 5 days after intrastromal injection of pSecLum or empty vector control plasmid DNA

Lumican levels increase as expected following intrastromal injection of pSecLum plasmid DNA. Keratocan levels also increase 2-fold after injection of pSecLum plasmid DNA, as compared with the empty vector control.

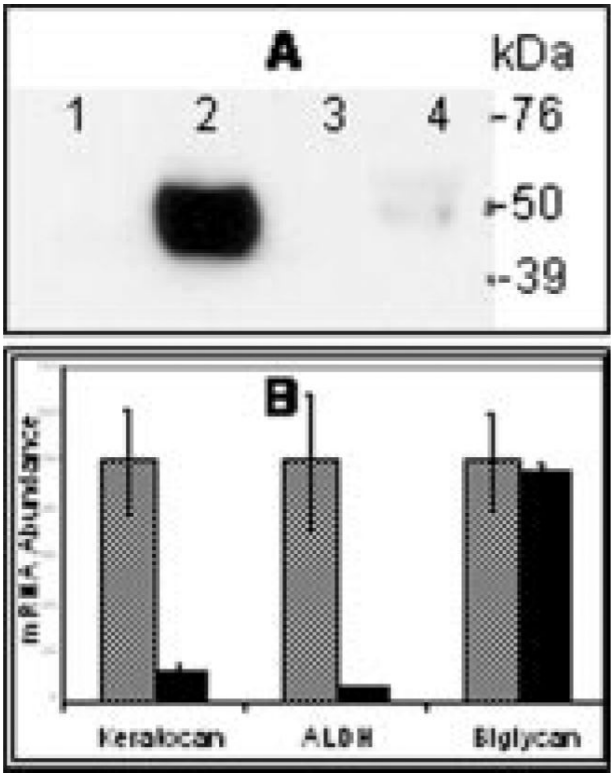

Fig. 6. Down-regulated expression of keratocan by bovine keratocytes treated with lumican siRNA

A, lumican was detected by Western blotting (as described under "Experimental Procedures") in culture medium (*lanes 2* and *4*) or cell lysates (*lanes 1* and *3*) of primary cultures of bovine keratocytes that had been transfected (*lanes 3* and *4*) with siRNA to bovine lumican or mock-transfected controls (*lanes 1* and *2*) 96 h after transfection. Transfection of siRNA significantly suppresses the synthesis of lumican. *B*, quantitative real time RT-PCR was used to determine relative mRNA pools in primary keratocytes 72 h after lumican siRNA transfection (*solid bars*) or mock transfection (*patterned bars*) using primer/probes for keratocan, aldehyde dehydrogenase (*ALDH*), and biglycan as previously described (39).

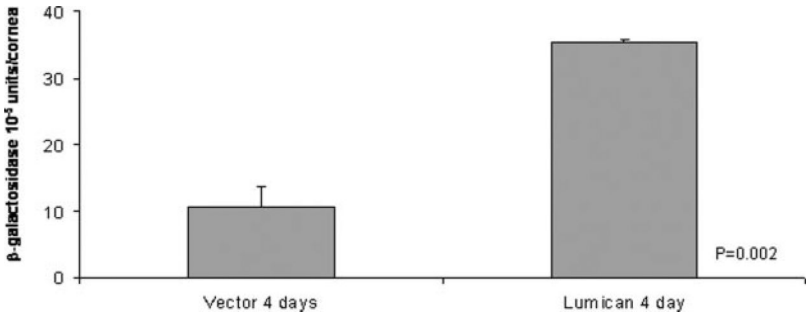

Fig. 7. Keratocan promoter activity assay performed in *Lum*$^{-/-}$ animals measuring the ability of the keratocan promoter to drive *β*-GEO expression in the presence or absence of lumican

The ability of an empty vector (*Vector*) or a lumican-expressing plasmid DNA construct (*Lumican*) to influence the expression of a *β*-GEO reporter gene by the keratocan promoter was measured 4 days after intrastromal injection. The presence of lumican significantly increases the activity of the keratocan promoter as shown in *β*-galactosidase 10^{-6} units/cornea as compared with vector control.

Table I

Mean corneal epithelial thickness, stromal thickness, and light scattering with the S.D. is shown for nontransgenic and Kera-LumWT founder lines 38 and 5 as measured by CMTF

Overall, there was no significant difference in epithelial or stromal thickness between nontransgenic and transgenic animals. The amount of light scattering measured in U_{AUC}, indicative of corneal haze, appears greater in transgenic as compared with nontransgenic animals.

Animal type	Epithelium	Stroma	Haze
	μm	*μm*	U_{auc}
Nontransgenic	48.42 ± 3.29	52.33 ± 5.46	565.26 ± 90.14
Kera-Lum38	46.27 ± 2.99	53.26 ± 3.23	696.73 ± 92.21
Kera-Lum5	45.42 ± 1.86	59.19 ± 5.58	643.42 ± 47.30