Testican-1 Promotes Resistance against *Pseudomonas aeruginosa*–Induced Keratitis through Regulation of MMP-2 Expression and Activation

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Purpose. Testican-1 (or SPOCK) is a highly conserved chimeric proteoglycan encoded by the *SPOCK1* gene. Protease regulatory activity has recently been demonstrated by this molecule and its family members testican-2 and -3. The present study tested the hypothesis that testican-1 regulates corneal matrix metalloproteinase (MMP)-2 expression, thus improving disease outcome after *Pseudomonas aeruginosa*–induced keratitis.

Methods. C57BL/6 (B6) and BALB/c mice were routinely infected with *P. aeruginosa* and were evaluated at various postinfection (pi) times for corneal expression of testican-1 and MMP-2, by PCR array, real-time RT-PCR, ELISA, activity assays, zymography, and immunohistochemistry. Next, B6 mice were treated with recombinant human (rh) testican-1, and expression was knocked down in BALB/c ice by siTestican-1 treatment, to determine the relationship between the two molecules.

Results. BALB/c versus B6 mice expressed significantly higher mRNA and protein levels of testican-1 after *P. aeruginosa*-induced ocular infection. MMP-2 expression and activation was also disparate between the two mouse strains. After rhTestican-1 treatment in B6 mice, overall disease response was significantly improved, whereas siRNA treatment of BALB/c mice converted the normally resistant response to susceptible. Testican-1 was shown to influence MMP-2 expression, activation, and regulation, as well.

Conclusions. This study demonstrates corneal expression of testican-1 and its temporal regulation of MMP-2 expression and activation after induction of bacterial keratitis. Furthermore, the data collectively indicate that testican-1 is a novel target for disease treatment to promote better disease outcome regarding chronic inflammation and infection and diseases involving pathologic tissue destruction. (*Invest Ophthalmol Vis Sci.* 2011;52:5339–5346) DOI:10.1167/iovs.10-6920

In regard to the eye, exposure to infectious agents may lead to complications that consist of structural alterations to the cornea. Bacterial infections caused by *Pseudomonas aeruginosa* and other Gram-negative bacteria typically present as a rapidly progressing, suppurative stromal infiltrate with marked mucopurulent exudate. Necrosis, accompanied by inflammatory epithelial edema and stromal ulceration, is also characteristic of bacterial keratitis and can culminate in significant stromal tissue destruction and loss of function. The progressive disruption and destruction of extracellular matrix (ECM) components are morphologic hallmarks that are not limited to the pathogenesis of bacterial keratitis, but are also pertinent to pathologic conditions associated with chronic inflammation and infection and disease states for numerous tissue sites including lung, heart, and connective tissue.

Matrix metalloproteinases (MMPs) comprise a family of Zn^{2+}-dependent endopeptidases that function through proteolytic activity to maintain and remodel tissue architecture.[1] They are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs), and MMP substrates are composed of a variety of ECM components, growth factors, secreted cytokines/chemokines, and cell surface molecules. MMPs are critical in a wide range of biological processes, including embryonic development and tissue repair and regeneration.[2] However, these molecules also have been linked to destructive processes and human disease, including as corneal infection, chronic obstructive pulmonary disease, tumor invasion/metastasis, and multiple sclerosis.[3,4] Substantial information has been garnered through sequence determination, protein analysis, and genetic approaches using single or complex deletions of MMP genes regarding the various roles and temporal and spatial localization of MMPs during disease states. Studies have revealed that many MMP substrates can also serve to regulate MMP expression in a coordinated effort to ensure efficient activation and control of MMPs.[5–7] However, the in vivo complexities regarding MMP regulation of substrate function prevail and regulation of MMP expression and activation remains, in large part, unclear.

Testican is a highly conserved chimeric proteoglycan that was initially identified in human seminal plasma.[8] It is also known by the term SPOCK, reflecting the modular organization of its core protein, which includes four SPARC/osteonectin domains and both CWCV and Kazal-like domains, which are present in thyropin-type cysteine protease inhibitors. The testican subgroup is part of the larger BM-40/SPARC/osteonectin protein family and includes three members (testicans-1, -2, and -3), all of which contain an extracellular calcium-binding domain and a follistatin-like domain. Thus far, studies have demonstrated that testican-1 plays a role in the regulation of brain development by blocking substrate attachment and neurite outgrowth of neuronal cells.[9,10] It was also thought that, apart from the testis, testican expression was limited to the brain and nervous system—mainly in pyramidal cells of the hippocampus. However, subsequent studies have revealed that testican is expressed by endothelial cells and chondrocytes. In relationship to MMPs, testican-1 and -3 have been demonstrated in vitro to inhibit activation of pro-MMP-2 via upstream inhibition of either membrane type (MT)1-MMP or MT3-MMP, although testican-2 abrogates the inhibition of MT-MMPs, thus leading to

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MMP-2 activation.[11] These data provoke closer examination concerning a role for testican in modulating ECM degradation and/or restoration.

As such, in the present study, we utilized well-characterized murine models of inflammatory disease wherein corneal infection induced experimentally by *P. aeruginosa* causes corneal perforation in C57BL/6 (B6) mice, classified as susceptible, whereas the infection effectively resolves in BALB/c mice, classified as resistant (no corneal perforation).[12] This study is the first to demonstrate the presence of testican-1 in the cornea with the use of these models and, furthermore, to illustrate a regulatory role for testican-1 regarding MMP-2 expression and activation using an in vivo animal model of disease.

METHODS

Mice

Female, 8-week-old B6 and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in accordance with National Institutes of Health guidelines. Humane animal care conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Infection

P. aeruginosa strain 19660, purchased from the American Type Culture Collection (ATCC, Manassas, VA), was prepared as previously described.[13] Mice (n = 5/group/time) were anesthetized with ethyl ether, and the left central cornea was scarified with a 25–5/8-gauge needle to create three 1-mm incisions penetrating no deeper than the superficial corneal stroma. A 5-μL aliquot of bacterial suspension containing 1.0×10^6 CFU/μL was topically applied to the wounded corneal surface, and disease response was graded at 1, 3, 5, and 7 days after infection (pi) with an established grading scale.[13] End points (5 and 7 days pi) for each set of experiments were based on corneal perforation of either the control or experimental group of mice.

siRNA Treatment

In correlative experiments, BALB/c mice (n = 5/group) were injected subconjunctivally with 5 μL containing 8 μM siRNA of testican-1 (Santa Cruz Biotechnology, Santa Cruz, CA), 1 day before infection. Next, the mice were treated topically with 5 μL of 4 μM siRNA, once on the day of infection (d 0) and twice 1 day pi. Control mice were similarly treated with a nontargeting scrambled siRNA. For TIMP-2 gene-silencing experiments, BALB/c mice were similarly injected and topically treated with TIMP-2 siRNA (Santa Cruz Biotechnology) and compared with scrambled siRNA–treated mice as controls. Testican-1 and TIMP-2 siRNAs are each composed of a pool of three target-specific 20 to 25 nt siRNAs designed to knock down gene expression.

Recombinant Human Testican-1 Treatment

One day before infection, B6 mice (n = 5/group) received subconjunctival injections (5 μL) containing 1 μg recombinant human (rh)Testican-1 (R&D Systems, Minneapolis, MN). The mice subsequently received IP injections containing 1 μg rhTestican-1 in 100 μL PBS on days 1 and 3 pi. Control mice were similarly treated with PBS containing 0.1% BSA. Regarding the use of recombinant human protein, there is 95% identity between cDNAs from the human testis and mouse brain, predicting a conserved function.[14]

Immunostaining for Testican-1

Normal, uninfected, and infected eyes of B6 and BALB/c mice were enucleated (n = 3/group/time) at 1, 3, and 5 days pi, immersed in PBS, embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer-thick sections were cut, mounted to poly-L-lysine-coated glass slides, incubated at 37°C overnight, and fixed in acetone. After blocking with 0.01 M PBS containing 2.5% BSA and donkey IgG (1:100) for 30 minutes at room temperature, sections were incubated with primary antibody, goat anti-testican-1 (1:100; Santa Cruz Biotechnology) for 1 hour; followed by Alexa Fluor 594–conjugated donkey anti-goat antibody (1:1500, Invitrogen, Carlsbad, CA) for an additional hour. Sections were then incubated for 2 minutes with nuclear stain (1:15,000; SYTOX Green; Lonza, Walkersville, MD). Controls were similarly treated with species-specific IgG in lieu of the primary antibody. Sections were visualized and digital images captured with a confocal laser scanning microscope (TSC SP2; Leica Microsystems, Exton, PA).

Real-Time RT-PCR

Normal, uninfected, and infected corneas (n = 5/group/time) from B6, BALB/c, and siRNA-treated mice were removed at 1, 3, 5, and 7 days pi. Corneas were harvested, placed in RNA isolation reagent (STAT-60; Tel-Test, Friendsville, TX), and total RNA was extracted (per the manufacturer's instructions) and used to produce a cDNA template for PCR amplification, as previously described.[15] Transcript levels for testican-1 and MMP-2 was detected by real-time RT-PCR with a single-color real-time RT-PCR detection system (MyiQ; Bio-Rad, Hercules, CA). PCR arrays and primers for testican-1 were purchased from SuperArray Bioscience Corporation (Frederick, MD); primers for MMP-2 were designed on computer (PrimerQuest SM; Integrated DNA Technologies, Coralville, IA) with the following primer sequences: MMP-2 (F) ACACTGGGACCTGTCACTCC and (R) CCAAATAAACCGGTCCTTGA. Relative mRNA levels were calculated using the relative standard curve method that compares the amount of target normalized to an endogenous reference, β-actin, using routine methods.[15]

MMP-2 ELISA and Zymography

Corneas from B6, BALB/c, siRNA-treated and recombinant protein-treated mice were analyzed for total (free, unbound) protein level of MMP-2 (ELISA) and/or MMP-2 proteolytic activity (zymography). Corneas from infected mice (n = 5/group/time) were harvested at 1, 3, 5, and 7 days pi. For ELISAs, whole corneas were individually homogenized with a glass microtissue grinder in 500 μL PBS containing 0.1% Tween-20, and centrifuged at 13,000g for 10 minutes. Supernatants were run in triplicate for MMP-2 (50 μL/well) per the manufacturer's instructions (R&D Systems). The reported sensitivity of this assay was <0.20 ng/mL. Regarding MMP-2 zymography, whole corneas were pooled and homogenized in 250 μL lysis buffer, centrifuged at 10,000g for 10 minutes at 4°C. Protein concentration was determined using the Bradford protein assay (Quick Start; Bio-Rad). Samples were diluted in sample buffer to appropriate concentrations (recombinant MMP-2 controls at 0.5 ng/lane and corneal samples at 10 μg/lane), then loaded onto 10% polyacrylamide gels containing 0.1% gelatin. The gels were run for 90 minutes at 125 V, and the separated enzymes were renatured by incubating the gels in renaturing buffer for 30 minutes at room temperature to remove the SDS, followed by developing buffer (or 50 mM Tris-HCl buffer [pH 8.0], containing 10 mM EDTA as a negative control for MMP activity) at 37°C overnight (16–20 hours) for gelatin digestion. To visualize activated MMP-2, gels were stained with Coomassie blue (SimplyBlue Safestain; Invitrogen) according to the manufacturer's instruction. To further confirm that the bands were metalloproteinases, separate gels were incubated at 37°C overnight in buffer lacking calcium and containing 20 mM EDTA, then stained and destained as described above.

Testican-1 Protein Analysis

For dot blot and Western blot analyses of tissue lysate, whole corneas from siTIMP-2-treated BALB/c mice were processed in lysis buffer under normal conditions and at 1 and 5 days pi and protein concentration was determined as described earlier. For dot blot, samples (10 μg) were pipetted onto nitrocellulose membrane, then blocked for 30 minutes in 5% solution of BSA prepared with TBST (1× TBS containing 0.05% Tween 20; Bio-Rad). Next, the blots were probed with primary goat anti-testican-1 (1:100) antibody for 1 hour (or HRP-conjugated

mouse anti-β-actin (1:200) as an internal control; Santa Cruz Biotechnology) washed three times for 5 minutes each with TBST, followed by incubation for 1 hour with donkey anti-goat IgG-HRP secondary antibody (1:2000) and developed using enhanced chemiluminescence (ECL Plus; GE Healthcare, Piscataway, NJ) according to the manufacturer's instruction. Blots were visualized by transillumination and quantitated using a gel documentation system (AlphaImager 2000 Documentation and Analysis System; Alpha Innotech Corp., San Leandro, CA). Integrated density values (IDVs) for each sample were corrected for the amount of internal control, β-actin, in each sample.

Regarding Western blot, corneal protein samples (20 μg) were separated on a 12.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane, blocked overnight at 4°C in 5% BSA. The blots were similarly probed as above with primary goat anti-testican-1 (1:100) antibody overnight, washed five times for 5 minutes each with TBST, followed by incubation for 1 hour with donkey anti-goat IgG-HRP secondary antibody (1:2000) and developed using the ECL method. Human recombinant testican-1 (95% homology with mouse testican-1; R&D Systems) served as the positive control. Testican-1 blocking peptide served as the negative control (Santa Cruz Biotechnology).

Statistical Analysis

The difference in clinical score between two groups at each time point was tested by the Mann-Whitney U test. An unpaired Student's *t*-test was used to determine the significance of real-time RT-PCR (except PCR array) and protein assays. Data were considered significant at $P < 0.05$. All experiments were repeated at least twice (except PCR array), and representative data from a typical experiment are shown unless otherwise noted.

Results

MMP-2 Expression in BALB/c versus B6 Mice

To investigate the mechanisms by which corneas of BALB/c mice are able to more efficiently and effectively heal after *P. aeruginosa*-induced corneal infection, whereas B6 corneas typically perforate, we profiled mRNA levels of 84 genes related to the extracellular matrix and adhesion molecules (RT^2 Profiler PCR Array; SA Biosciences-Qiagen, Frederick, MD). Results indicated that MMP-2 was elevated in the cornea of BALB/c over B6 mice at 3, 5, and 7 days pi (Table 1). Given the role of MMP-2 during the process of corneal healing (specifically, collagen remodeling via secretion of collagenase, and gelatinase B activity),[1] we further examined this particular protease in individual corneal samples by real-time RT-PCR under normal conditions and at 1, 3, 5, and 7 days pi in BALB/c and B6 mice. MMP-2 was significantly upregulated in BALB/c cornea when compared with B6 at 3, 5, and 7 days pi ($P = 0.03$, < 0.01, 0.01; Fig. 1A).

To confirm these data, protein levels of MMP-2 in the cornea were examined by using ELISA (total protein) and zymography (proteolytic activity); the results are provided in Figures 1B and 1C, respectively. MMP-2 protein expression, as detected by ELISA, in the B6 and BALB/c mouse corneas was assessed under normal conditions and at 1, 3, 5, and 7 days pi.

Table 1. PCR Array Analysis

Days PI	MMP-2	TIMP-2	Testican-1
1	1.1	1.32	3.49
3	1.7	3.39	12.66
5	1.52	1.92	11.89
7	2.23	3.25	7.06

Data are the fold difference of BALB/c versus B6.

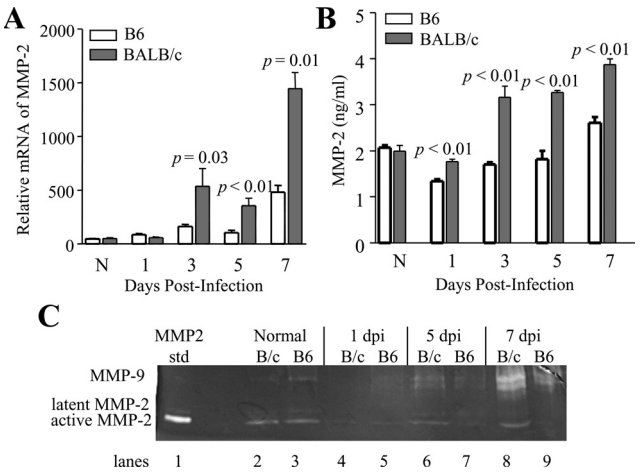

Figure 1. MMP-2 levels in the cornea in B6 versus BALB/c mice after ocular infection. (**A**) MMP-2 transcript levels were significantly elevated in BALB/c versus B6 mice at 3, 5, and 7 days pi. N, normal, uninfected cornea (N, $P = 0.59$; 1 day pi, $P = 0.06$). (**B**) MMP-2 total protein levels were significantly higher at 1, 3, 5, and 7 days pi in resistant BALB/c when compared to those in susceptible B6 mice. (N, $P = 0.5$). (**C**) Activity for MMP-2 appeared upregulated in BALB/c mice at 5 and 7 days pi; however, levels were comparable to B6 mice under normal (uninfected) conditions and at 1 day pi.

Results at the protein level corroborated those obtained at the transcript level, wherein MMP-2 protein expression in the cornea was significantly elevated in BALB/c versus B6 mice at all time points pi ($P < 0.01$).

Zymography (Fig. 1C) demonstrated that the normal corneas of the BALB/c and B6 mice (Fig. 1C, lanes 2 and 3, respectively) had detectable levels of active MMP-2 at the protein concentration tested. At 1 day pi, MMP-2 was detectable at very low levels in both groups of mice (Fig. 1C, lanes 4 and 5). By 5 and 7 days pi, the BALB/c mice (Fig. 1C, lanes 6 and 8) showed higher MMP-2 activity, whereas levels appeared remarkably reduced in corneas of the B6 mice (Fig. 1C, lanes 7 and 9). Negative controls (incubated with 10 mM EDTA) did not show any detectable bands for MMP-2 (data not shown).

Testican-1 Expression in BALB/c versus B6 Mice

Since MMP-2 expression and activation are markedly different between the two mouse strains, and previous in vitro studies suggest a regulatory role for testican-1 over MMP-2, this proteoglycan was examined as a modulator of the MMP-2-driven component of disease outcome after *P. aeruginosa*-induced ocular infection. Initially, the profiler PCR array data revealed that testican-1 corneal mRNA levels were highly upregulated in BALB/c over B6 mice at 1, 3, 5, and 7 days pi (Table 1).

Next, testican-1 was examined individually using real-time RT-PCR, and transcript levels were significantly elevated at 3, 5, and 7 days pi in corneas of the BALB/c versus the B6 mice (Fig. 2A); however, no significant differences were detected at 1 day pi or in the normal cornea. These data were further confirmed by testican-1 immunostaining at 1, 3, and 5 days pi (Fig. 2B). Control sections under normal (uninfected) conditions (data not shown) and at 1 day pi (top) were negative for testican-1 staining in both the B6 and BALB/c mice, but both groups stained positive for green nuclear stain (SYTOX; Lonza). While testican-1 (red) was evident in the corneal epithelium of B6 mice at 3 and 5 days pi, corneas of BALB/c mice revealed positive stromal staining, in addition to epithelial staining, at the same time points. Control sections (bottom panels) were similarly incubated, but in the absence of the primary antibody (replaced with goat IgG), and were negative

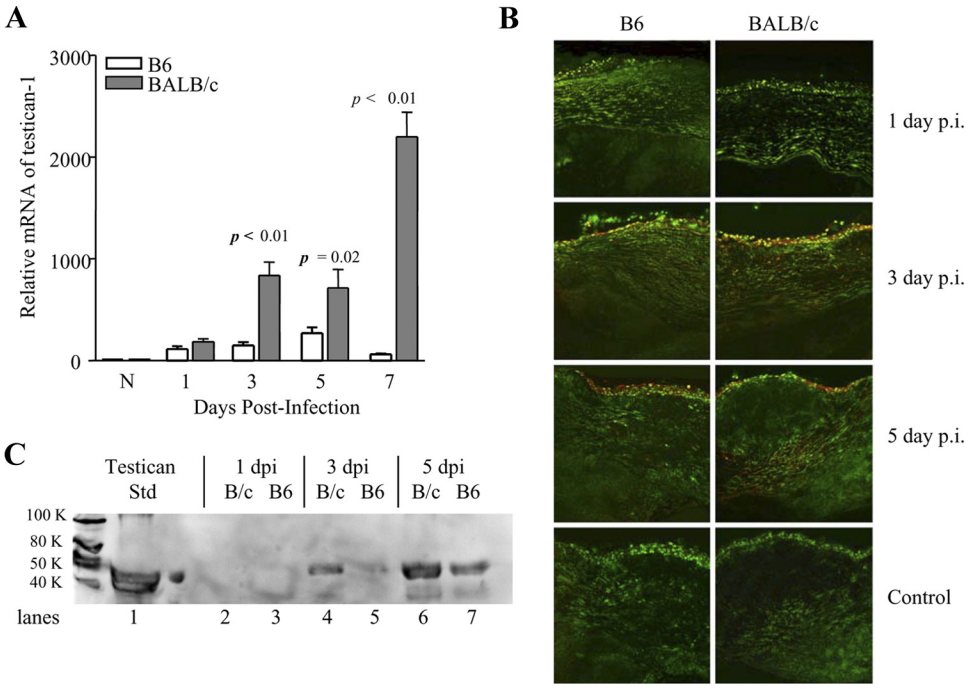

FIGURE 2. Testican-1 expression as detected from corneas of B6 and BALB/c mice after *P. aeruginosa* ocular infection. (**A**) Normal, uninfected corneas of the B6 and BALB/c mice showed comparable mRNA levels of testican-1 ($P = 0.9$). At 1 day pi, the BALB/c mice showed an increase in testican-1 expression ($P = 0.11$) that became significant at 3, 5, and 7 days pi compared to that in the B6 mice. (**B**) Immunostaining of testican-1 protein in corneas of the B6 (*left*) and BALB/c (*right*) mice was not detected under normal, uninfected conditions (data not shown) nor at 1 day pi. Positive testican-1 staining was evident in the B6 mice at 3 and 5 days pi, localized primarily to the corneal epithelium. Epithelial staining and positive stromal staining were revealed in the cornea of BALB/c mice at the same time points. Controls (primary antibody omitted) stained positive for green nuclear stain only. Magnification, ×50. (**C**) Western blot analysis revealed that testican-1 protein was not detectable in the BALB/c or the B6 mice at 1 day pi (*lanes 2* and *3*), whereas expression appeared greater in the BALB/c (*lanes 4* and *6*) versus the B6 mice (*lanes 5* and *7*) at both 3 and 5 days pi. Human recombinant testican-1 (MW, 50,000–56,000) served as the positive control (*lane 1*).

for testican-1 immunostaining and positive for green nuclear stain (SYTOX; Lonza). In addition to immunostaining, corneal expression of testican-1 was detected by Western blot at 1, 3, and 5 days pi (Fig. 2C). Results showed that testican-1 was not detectable in either the BALB/c or the B6 mice at 1 day pi (lanes 2 and 3, respectively); however, at both 3 and 5 days pi, the BALB/c mice revealed greater protein expression (lanes 4 and 6) when compared to B6 (lanes 5 and 7) at the same time points. These results correlated well with MMP-2 levels. Not only were testican-1 and MMP-2 levels increased over time, but both were upregulated in BALB/c versus B6 mice, as well.

siTestican-1 Treatment of BALB/c Mice

Given the divergent expression of testican-1 in resistant BALB/c versus susceptible B6 mice, as demonstrated at the mRNA (real-time RT-PCR) and protein (immunostaining and Western blot) levels, testican-1 expression was silenced in resistant BALB/c mice to test the role of this proteoglycan in disease pathogenesis. Verification of testican-1 silencing in the cornea of BALB/c mice was performed by real-time RT-PCR (Fig. 3D). Results indicated that siRNA treatment significantly inhibited testican-1 mRNA expression through 7 days pi when compared to the scrambled siRNA–treated BALB/c mice.

Disease progression was observed in scrambled siRNA–treated BALB/c mice (control) and documented through 7 days pi by clinical score and photography with a slit lamp (Figs, 3A–C). Knockdown of testican-1 gene expression resulted in poorer disease outcome, as indicated by clinical score (Fig. 3A); corneas were significantly worse at 3, 5, and 7 days pi ($P < 0.01$), ultimately perforating (grade = +4) compared to control corneas (grade = +2). Photographs taken by slit lamp showed that treatment with siTestican-1 (Fig. 3B) exacerbated the pathologic condition, as indicated by increased corneal opacity and perforation when compared with that in the scrambled siRNA–treated control mice (Fig. 3C).

To test whether testican-1 regulates corneal MMP-2 during bacterial keratitis, mRNA, we analyzed total free protein and activity levels of MMP-2 over time by real-time RT-PCR, ELISA, and Western blot in siTestican-1-treated versus scrambled siRNA–treated (control) BALB/c mice (Figs. 3E–G). After testican-1 silencing in the BALB/c mice, corneal MMP-2 mRNA levels (Fig. 3E) were similar to those in the scrambled controls at 1 day pi, but then were significantly downregulated at 3, 5, and 7 days pi ($P < 0.01$). In parallel, MMP-2 total protein levels (Fig. 3F) were significantly downregulated at 3, 5, and 7 days pi ($P < 0.001$) after siRNA treatment when compared with the scrambled controls. Furthermore, MMP-2 activity levels also were reduced in corneas of siTestican-1 versus scrambled BALB/c mice at 1, 3, and 5 days pi, as shown in Figure 3G. No difference was observed in normal, uninfected corneas (Fig. 3G, lanes 2 and 3).

rhTestican-1 Treatment of B6 Mice

To parallel testican-1 silencing in resistant BALB/c mice, we next treated susceptible B6 mice with rhTestican-1 and similarly examined the effects. Clinical score and slit lamp photography (Figs. 4A–C) were used to document disease progression through 5 days pi and compared with that in the PBS/BSA-treated B6 mice (control). Treatment with rhTestican-1 resulted in significantly improved disease outcome at 3 and 5 days pi ($P < 0.01$), as indicated by clinical score (Fig. 4A). Photographs taken by slit lamp demonstrate treatment with rhTestican-1 (Fig. 4C) ameliorated disease pathogenesis as indicated by decreased corneal opacity when compared with that in the PBS/BSA-treated controls (Fig. 4B).

Next, we examined the effects of rhTestican-1 treatment regarding corneal MMP-2 expression/activation during bacterial keratitis. Transcript and activity levels of MMP-2 were analyzed over time by real-time RT-PCR and gelatin zymography in the recombinant-treated versus the PBS/BSA-treated (control) B6 mice (Figs. 4D, 4E). After recombinant treatment in the B6 mice, corneal MMP-2 mRNA levels (Fig. 4D) were significantly elevated at 3 and 5 days pi ($P = 0.04$) when compared to the controls. In addition, MMP-2 activity (Fig. 4E)

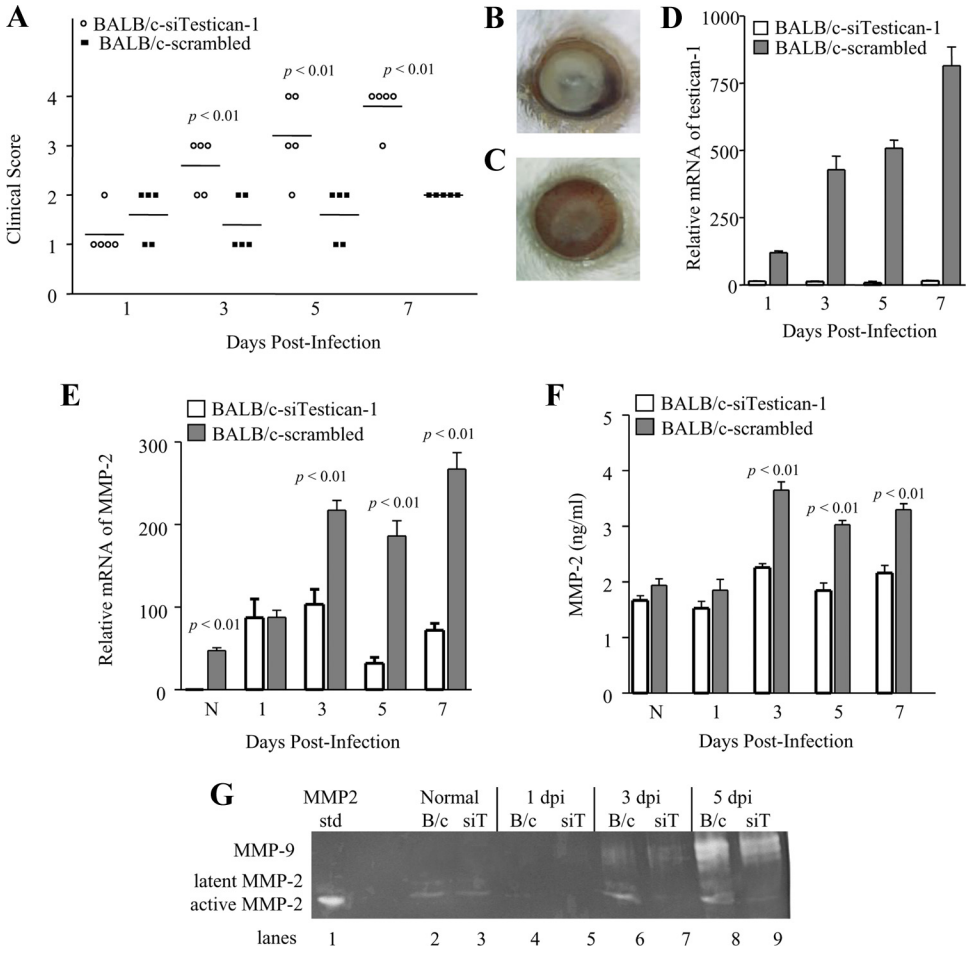

FIGURE 3. Disease response and corneal MMP-2 expression/activity in siTestican-1 treatment versus scrambled siRNA control in BALB/c mice after *P. aeruginosa* ocular infection. The clinical score indicated that silencing of testican-1 significantly exacerbated disease response at 3, 5, and 7 days pi (1 day pi, $P = 0.31$). (**A**) Converting the typical response from resistant to susceptible. Photographs taken by slit lamp of *P. aeruginosa*-infected BALB/c eyes are shown after treatment with siTestican-1 (**B**) or scrambled siRNA (**C**). Representative eyes were photographed for both groups at 7 days pi. Magnification, ×5. Verification of testican-1 silencing (**D**) was performed through 7 days pi and revealed significantly reduced corneal transcript levels at all time points when compared to the scrambled siRNA control BALB/c mice. MMP-2 mRNA levels (**E**) were significantly lower under normal conditions and at 3, 5, and 7 days pi in the siTestican-1-treated mice when compared to the scrambled siRNA BALB/c controls; however, the levels were comparable to those in the controls at 1 day pi ($P = 0.31$). Protein levels for MMP-2 (**F**) corresponded with mRNA levels, which were significantly reduced at 3, 5 and 7 days pi (N, $P = 0.9$; 1 day pi, $P = 0.26$). In addition, MMP-2 activity (**G**) appeared reduced at 3 and 5 days pi after siTestican-1 treatment (siT) when compared to controls.

was elevated at both 1 and 5 pi (lanes 5 and 7); however, there appeared to be no difference in the normal, uninfected corneas after rhTestican-1 treatment (lane 3) when compared to the controls (lane 2).

siTIMP-2 Treatment and Testican-1/MMP-2 Levels

We next examined the mechanism for disparate testican-1 expression levels in BALB/c versus B6 mice. It has been hypothesized that TIMP-2 levels dictate MT1-MMP substrates in an in vitro cell culture model.[4] In the resistant BALB/c mouse, the PCR profile array revealed that TIMP-2 levels were two- to threefold higher than in the B6 mice after *P. aeruginosa*-induced ocular infection (Table 1). Therefore, we investigated the potential regulatory role of TIMP-2 over testican-1 by silencing TIMP-2 in the BALB/c mice.

The clinical score (Fig. 5A) indicated poorer disease outcome after siTIMP-2 treatment, but the difference was not significant when compared with the scrambled controls. Silencing was confirmed by real-time RT-PCR through 7 days pi (Fig. 5B). Protein levels for testican-1 were not significantly different after siTIMP-2 treatment when compared with scrambled siRNA BALB/c controls under normal conditions or at 1 and 5 days pi (Fig. 5C) when detected by dot blot analysis. However, when visualized by Western blot, siTIMP-2 treatment revealed double bands for testican-1 under normal (uninfected) conditions and at both 1 and 5 days pi indicating cleavage/degradation, whereas only a single band indicative of intact protein (MW of testican-1: 50,000–56,000) appeared in scrambled siRNA BALB/c controls (Fig. 5D). In addition, MMP-2 total protein levels were significantly downregulated after siTIMP-2 treatment when compared with the controls at 3, 5, and 7 days pi (Fig. 5E). These results were further corroborated by zymography (Fig. 5F), wherein at 5 days pi, active MMP-2 levels were decreased (but increased latent MMP-2) after siTIMP-2 treatment (lane 7) when compared to scrambled BALB/c controls (lane 6). No differences were observed between the two groups at 1 day pi (lanes 4 and 5) or under normal (uninfected) conditions (lanes 2 and 3).

DISCUSSION

Degradation of the matrix facilitates inflammatory cell transmigration into the infected tissue site, which is imperative in mounting an effective inflammatory/immune response. However, reconstitution of the ECM and restoration of tissue homeostasis are equally essential components of a successful host response to infection or disease. Progressive degradation of extracellular matrices is a fundamental characteristic of chronic inflammation and pathologic disease states. In fact, it is the unregulated, persistent proteolysis of the ECM that is a major cause of bystander tissue damage, as illustrated by the disease pathogenesis of B6 mice during corneal infection.[12] In the present study, we examined mechanisms by which stromal destruction predominates in the B6 cornea, whereas stromal healing and reorganization occurs in the cornea of BALB/c mice.

It is well understood that the enzymatic activity of MMPs largely contribute to modulating normal, as well as pathologic, conditions of the ECM.[16] During ocular wound healing, MMP expression and activation are important in basement mem-

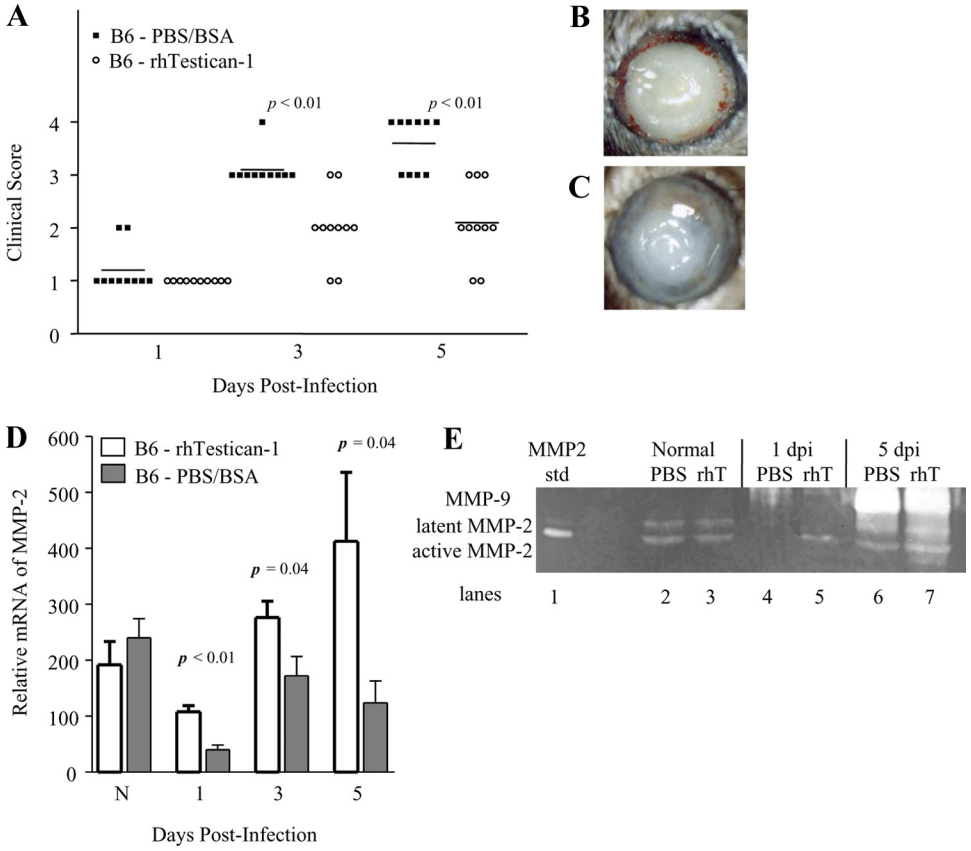

FIGURE 4. Disease response in rhTestican-1 treatment versus PBS/BSA-treated control in B6 mice after *P. aeruginosa* ocular infection. Clinical score demonstrated that treatment with rhTestican-1 significantly improved disease outcome at 3 and 5 days pi (**A**), converting the typical response from susceptible to resistant (1 day pi, $P = 0.68$). Photographs (taken by slit lamp) of *P. aeruginosa*-infected B6 eyes are shown after treatment with PBS/BSA (**B**) or rhTestican-1 (**C**). Representative images were taken for both groups at 5 days pi. Magnification, ×5. Transcript levels of MMP-2 (**D**) were significantly upregulated at 1, 3, and 5 days pi after rhTestican-1 treatment when compared with the controls (N, $P = 0.29$). MMP-2 activity (**E**) appeared elevated at both 1 and 5 days pi after rhTestican-1 treatment (rhT) when compared with the controls (PBS), whereas there appeared to be no difference between the groups under normal conditions.

brane remodeling, wound contraction, debris clearance, and fibrotic repair tissue deposition.[1] Complementary to, yet independent of, proteolytic activity of these molecules, MMPs can influence molecular mediators of inflammation through the generation of both pro- and anti-inflammatory signals.[1] MMP-7 has been shown to have a protective response in the small intestine by proteolytic processing of the mature form of α-defensin,[17] an antimicrobial peptide expressed by Paneth cells. MMP-2, -3, -7, and -9 have been shown to release TGF-β from the ECM, allowing for its subsequent activation[18,19] and execution of immunomodulatory functions, which include selective stimulation of ECM components. This family of proteolytic enzymes has also been linked to both normal and pathologic neovascularization by regulating the bioavailability of angiogenic factors, such as vascular endothelial growth factor and epithelial growth factor.[1] A previous study from our laboratory showed that MMP-9 (gelatinase B) regulates corneal immune function by degrading collagen IV and upregulating chemotactic cytokines/chemokines IL-1β and macrophage inflammatory protein (MIP)-2.[20] Results from the present study demonstrate disparate expression profile for MMP-2 in the cornea of BALB/c versus B6 mice. This differential MMP expression is thought to influence matrix regeneration or degradation, thus potentiating resistance (BALB/c) or susceptibility (B6) after *P. aeruginosa*-induced ocular infection. When further explored, it was found that MMP-2 (gelatinase A), noted for its role in wound repair and development,[1,2,21] is upregulated late in BALB/c mice; thus, corresponding to a shift in the immune response toward wound healing and tissue restoration. The MMP-2 data corresponded with a recent study of MMP expression during fungal keratitis.[22] The investigators spatially localized MMP-2 expression to corneal epithelium using immunofluorescent staining and immunohistochemistry. Given that the BALB/c cornea remains more intact than B6 after bacterial keratitis, we hypothesize that during the wound-healing phase, higher MMP-2 levels are due to (1) direct protein expression by corneal epithelial cells or (2) the indirect production of epithelial-derived signals that augment MMP-2 expression. MMP-2 levels are lower in B6 mice since the corneal epithelium is, in large part, decimated, which strengthens the supposition that MMP-2 expression in the cornea is dependent on corneal epithelial cells and warrants additional investigation. In addition, given that MMP-2 is linked to corneal healing, remodeling, and maintenance, we surmise that the decrease in protein levels for both BALB/c and B6 mice at 1 day pi is an effort to clear the *Pseudomonas* infection, which is then followed by increased levels of MMP-2 focused on healing and reconstitution of the stroma.

As previously mentioned, MMP-2 activation can occur via interaction with MT1-MMP. Nakada et al.[11] have demonstrated in an in vitro cell culture model that testican-1 (and testican-3) suppresses MMP-2 activation via MT1-MMP inhibition. They also showed that testican-2 can abrogate this inhibition, leading to MMP-2 activation. In contrast, the data presented herein showed that resistant BALB/c mice expressed significantly higher levels of testican-1 and MMP-2 when compared to susceptible B6 mice, indicating that testican-1 does not inhibit MMP-2 activation in vivo. This finding is further supported by the spatial relationship between testican-1 and MMP-2, as demonstrated by immunofluorescent staining (Fig. 2B); testican-1 protein was limited to the epithelium of B6 mice, whereas it was detected throughout the epithelium and stroma of the BALB/c mice (spatially facilitating the effects on MMP-2 within the stroma). It could be hypothesized that testican-2 levels abate inhibitory effects of testican-1. However, when testican-1 was silenced in the resistant BALB/c mouse, disease severity was worsened and MMP-2 (as well as MMP-13, data not shown) mRNA and protein levels were significantly decreased over those in the controls, further supporting a direct relationship between testican-1 and MMP-2. Moreover, when susceptible

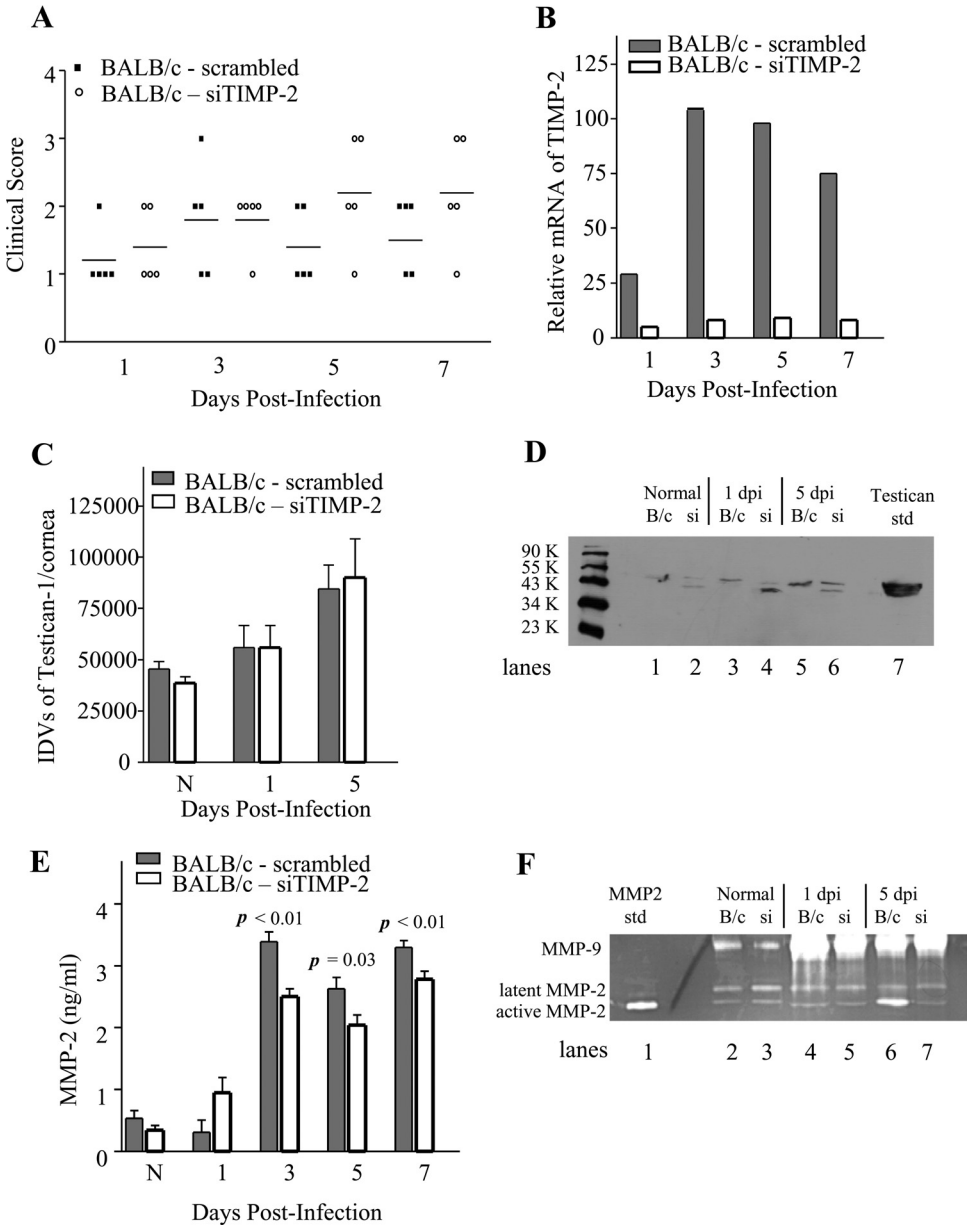

FIGURE 5. Effects of siTIMP-2 treatment on testican-1 and MMP-2 protein levels in BALB/c mice after ocular infection. (**A**) The clinical score showed that TIMP-2 silencing resulted in increased disease severity, but the difference was not significant when compared with the scrambled siRNA controls (1, 3, 5, and 7 days pi; $P = 0.69$, 0.99, 0.15, and 0.11, respectively). (**B**) Verification of testican-1 silencing was performed through 7 days pi and revealed significantly reduced corneal transcript levels at all time points when compared to scrambled control BALB/c mice. (**C**) IDVs from dot blot analysis demonstrated that silencing of TIMP-2 resulted in comparable testican-1 protein expression under normal conditions and at 1 and 5 days pi when compared to scrambled siRNA–treated control animals ($P = 0.33$, 0.5, and 0.52, respectively). There were no significant differences between any of the groups. (**D**) After siTIMP-2 treatment (si), Western blot analysis revealed double bands for testican-1 protein, indicating degradation/cleavage, under normal conditions and at 1 and 5 days pi (lanes 2, 4, and 6, respectively). Single bands, revealing intact protein (MW, 50,000–56,000), were detected for testican-1 in scrambled siRNA–treated mice at the same time points (*lanes 1*, *3*, and *5*, respectively). Human recombinant testican-1 served as the positive control (*lane* 7). (**E**) MMP-2 protein levels were significantly decreased at 3, 5, and 7 days pi after siTIMP-2 treatment when compared with control BALB/c mice (N, $P = 0.2$, 1 day pi, $P = 0.06$). (**F**) MMP-2 activity was similar to that in the controls under normal conditions and at 1 day pi; however it was markedly decreased at 5 days pi after siTIMP-2 treatment, as detected by zymography.

B6 mice were treated with rhTestican-1 protein, the disease progression was, in fact, reduced, and MMP-2 was significantly increased over the controls at both mRNA and protein levels. It is plausible that changes in MMP expression could be merely in response to changes in infection severity and subsequently, changes in the amount of viable ocular tissue, instead of testican-1 levels. However, it is important to note that these changes in MMP expression also occurred at earlier time points (3 days pi) when the amount of viable tissue between experimental and control groups were, in large part, comparable. In addition, gene silencing of TIMP-2 in resistant BALB/c mice did not significantly exacerbate disease outcome, yet MMP-2 levels were still decreased compared to the controls, further negating the idea that changes in MMP-2 expression are merely a result of disease severity.

Our results correlate with a model described by Kudo et al.,[4] wherein MT1-MMP substrates are determined by TIMP-2 levels. At low (or no) TIMP-2 levels, MT1-MMP does not activate MMP-2, yet directly digests its own substrates, which includes testican-1. At moderate levels, TIMP-2 forms a complex with MT1-MMP and allows MMP-2 activation to occur. However, when TIMP-2 levels are saturated, pro-MMP-2 transiently replaces TIMP-2 for activation, but free TIMP-2 then blocks active MMP-2. In the resistant BALB/c mouse, TIMP-2 levels were two to threefold higher than those in the B6 after *P. aeruginosa*-induced ocular infection. Given that TIMP-2 levels in susceptible B6 mice are low, this hypothesis provides a mechanism for reduction in activated MMP-2 levels (MT1-MMP does not complex with TIMP-2 to activate MMP-2) and lower testican-1 protein levels (due to MT1-MMP digestion) when compared with the resistant BALB/c mouse. In fact, when TIMP-2 was silenced in BALB/c mice, disease response was exacerbated, and MMP-2 protein levels were significantly decreased, resulting in an accumulation of latent MMP-2, when compared with that in the scrambled siRNA–treated controls. More notably, testican-1 protein levels were comparable between siTIMP-2-treated and controls groups; however, siTIMP-2 treatment revealed cleaved or digested testican-1 protein (multiple bands), whereas testican-1 protein remained intact in the corneas of the control animals. These data not only support

the hypothesis that MMP-2 activation is dependent on TIMP-2 levels, but that testican-1 serves as an alternate substrate for MT1-MMP. Although there are mechanisms at the transcriptional level that remain to be explored, these data support a mechanistic role of the differential expression of both testican-1 and MMP-2 at the protein level.

In regard to the eye, testican-1 expression has been detected throughout the corneal epithelium and stroma (as demonstrated herein), as well as the epithelial lining of both the cornea and conjunctiva.[23] However, as previously mentioned, vascular endothelial cells have also been implicated as a source for this proteoglycan. In our model of bacterial keratitis, greater neovascularization is observed in BALB/c versus B6 mice after infection (LDH, unpublished data, 2010). As such, this observation warrants further investigation into a potential correlation between neovascularization and testican-1 expression.

In summary, this study provides a new perspective on MMP function and regulation within the context of resistant and susceptible murine models of ocular infection. Moreover, its implications can be extended to other sites of inflammation and/or disease. The results demonstrate the temporal significance of MMP expression regarding wound healing. More significantly, however, they also delineate a regulatory role for testican-1; a role that was previously restricted to inhibition of MMP-2 in vitro, but is revealed in vivo to modulate MMP-2 activation and promote wound healing. MMPs remain intricate enzymes that are highly regulated, but to date no unifying regulatory theme has been identified. Testican-1 offers potential clinical relevancy in MMP-driven diseases and pathologic conditions, which warrants further investigation into its broad MMP regulatory properties.

References

1. Sivak JM, Fini ME. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. *Prog Retin Eye Res.* 2002; 21:1–14.
2. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* 2000;14(17):2123–2133.
3. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiol Rev.* 2007; 87:69–98.
4. Kudo T, Takino T, Miyamori H, Thompson EW, Sato H. Substrate choice of membrane-type 1 matrix metalloproteinase is dictated by tissue inhibitor of metalloproteinase-2 levels. *Cancer Sci.* 2007;98: 569–568.
5. Hollborn M, Stathopoulos C, Steffen A, Wiedemann P, Kohen L, Bringmann A. Positive feedback regulation between MMP-9 and VEGF in human RPE cells. *Invest Ophthalmol Vis Sci.* 2007;48(9): 4360–4367.
6. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood.* 2002;100: 1160–1167.
7. Van den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G. Gelatinase B/MMP-9 and neutrophil collagenase/ MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/ CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem.* 2003;270:3739–3749.
8. Bonnet F, Perin JP, Maillet P, Jolles P, Alliel PM. Characterization of a human seminal plasma glycosaminoglycan-bearing polypeptide. *Biochem J.* 1992;288:565–569.
9. Roll S, Seul J, Paulsson M, Hartmann U. Testican-1 is dispensible for mouse development. *Matrix Biol.* 2006;25:373–381.
10. Marr HS, Edgell CJ. Testican-1 inhibits attachment of Neuro-2a cells. *Matrix Biol.* 2003;22:259–266.
11. Nakada M, Miyamori H, Yamashita J, Sato H. Testican 2 abrogates inhibition of membrane-type matrix metalloproteinases by other testican family proteins. *Cancer Res.* 2003;63:3364–3369.
12. Hazlett LD. Corneal response to Pseudomonas aeruginosa infection. *Prog Retin Eye Res.* 2004;23(1):1–30.
13. Rudner XL, Kernacki KA, Barrett RP, Hazlett LD. Prolonged elevation of IL-1 in Pseudomonas aeruginosa ocular infection regulates macrophage-inflammatory protein-2 production, polymorphonuclear neutrophils persistence, and corneal perforation. *J Immunol.* 2000;164:6576–6582.
14. Bonnet F, Perin JP, Charbonnier F, et al. Structure and cellular distribution of mouse brain testican: association with the postsynaptic area of hippocampus pyramidal cells. *J Biol Chem.* 1996;271: 4373–4380.
15. Szliter EA, Lighvani S, Barrett RP, Hazlett LD. Vasoactive intestinal peptide balances pro- and anti-inflammatory cytokines in the Pseudomonas aeruginosa-infected cornea and protects against corneal perforation. *J Immunol.* 2007;178:1105–1114.
16. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta.* 2005;1755(1): 37–69.
17. Wilson CL, Ouellette AJ, Satchell DP, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science.* 1999;286:113–117.
18. Mohammed FF, Smookler DS, Khokha R. Metalloproteinases, inflammation, and rheumatoid arthritis. *Ann Rheum Dis.* 2006;62: 43–47.
19. Hojilla C, Mohammed F, Khokha R. Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. *Br J Cancer.* 2003;89:1817–1821.
20. McClellan SA, Huang X, Barrett RP, et al. Matrix metalloproteinase-9 amplifies the immune response to Pseudomonas aeruginosa corneal infection. *Invest Ophthalmol Vis Sci.* 2006;47:256–264.
21. Zhang JS, Bai S, Zhang X, Nagase H, Sarras MP Jr. The expression of gelatinase A (MMP-2) is required for normal development of zebrafish embryos. *Dev Genes Evol.* 2003;213(9):456–463.
22. Yuan XB, Mitchell M, Wilhelmus KR. Expression of matrix metalloproteinases during experimental Candida albicans keratitis. *Invest Ophthalmol Vis Sci.* 2009;50:737–742.
23. Turner HC, Budak MT, Akinci MA, Wolosin JM. Comparative analysis of human conjunctival and corneal epithelial gene expression with oligonucleotide microarrays. *Invest Ophthalmol Vis Sci.* 2007;48:2050–2061.