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Reduced Scleral TIMP-2 Expression Is Associated With Myopia Development: TIMP-2 Supplementation Stabilizes Scleral Biomarkers of Myopia and Limits Myopia Development

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PURPOSE. The purpose of this study was to determine the endogenous regulation pattern of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the tree shrew sclera during myopia development and investigate the capacity of exogenous TIMP-2 to inhibit matrix metalloproteinase-2 (MMP-2) in vitro and both scleral collagen degradation and myopia development in vivo.

METHODS. TIMP-2 expression in the sclera during myopia development was assessed using polymerase chain reaction. In vitro TIMP-2 inhibition of MMP-2 was investigated using a gelatinase activity plate assay and zymography. Tree shrews were injected with a collagen precursor before undergoing monocular form deprivation and concurrent daily subconjunctival injections of either TIMP-2 or vehicle to the form-deprived eye. In vivo ocular biometry changes were monitored, and scleral tissue was collected after 12 days and assayed for collagen degradation.

RESULTS. The development of myopia was associated with a mean reduction in TIMP-2 mRNA expression after 5 days of form deprivation ($P < 0.01$). Both activation and activity of MMP-2 were inhibited by TIMP-2 with an IC_{50} of 10 to 20 and 2 nM, respectively. In vivo exogenous addition of TIMP-2 significantly reduced myopia development ($P < 0.01$), due to reduced vitreous chamber elongation ($P < 0.01$). In vivo TIMP-2 treatment also significantly inhibited posterior scleral collagen degradation relative to vehicle-treated eyes ($P < 0.01$), with levels similar to those in control eyes.

CONCLUSIONS. Myopia development in mammals is associated with reduced expression of TIMP-2, which contributes to increased degradative activity in the sclera. It follows that replenishment of this TIMP-2 significantly reduced the rate of both scleral collagen degradation and myopia development.

Keywords: myopia, gene expression, collagen

It has long been known that excessive axial elongation of the ocular globe results in the development of myopia in humans and animal models of myopia.¹ High degrees of myopia carry greater risk of visual impairment that cannot be overcome by optical means, with high myopia consistently demonstrated to be one of the leading causes of vision loss.² Animal models of myopia have demonstrated that active remodeling of the ocular coat, principally involving collagen, facilitates the change in eye size.³⁻⁶ Previous studies in the tree shrew model of myopia have shown that scleral collagen content is reduced at the posterior pole of myopic eyes,⁷⁻¹⁰ arising from reduced collagen synthesis and increased collagen degradation.¹¹ The combination of these changes leads to thinning of the sclera, particularly at the posterior pole of the eye, and the sclera becomes more distensible under the biomechanical force of the intraocular pressure.¹²⁻¹⁴

Members of the matrix metalloproteinase (MMP) family and their natural regulators, the tissue inhibitor of MMPs (TIMPs),

have been implicated in this process of matrix turnover in the sclera of myopic eyes.¹⁵⁻¹⁹ MMPs are secreted as latent proenzymes that are activated through cleavage of the inhibitory amino terminal domain.²⁰ MMP-2, arguably the most studied metalloproteinase in the sclera, is capable of degrading a range of components of the scleral extracellular matrix, including collagens and proteoglycans.^{21,22} The active form of MMP-2 can subsequently be inhibited by its natural inhibitors, the TIMPs, such as TIMP-1 and TIMP-2, which bind the active enzyme directly.²¹ Thus, the balance of MMP-2 activation and inhibition is critical to normal scleral matrix turnover and, therefore, regulation of eye size. During myopia development, increased MMP-2 expression and activity is observed in both mammalian^{23,24} and avian^{16,25,26} models of myopia. Despite the mechanistic differences in scleral remodeling in these models, this increased activity of MMP-2 is associated with increased scleral collagen degradation during myopia development.¹¹ For these changes in MMP-2 levels and activity to occur, one or



more of the following processes is likely to be present: (1) increased MMP-2 expression; (2) increased MMP-2 activation; and (3) decreases in free TIMP-2 (and other free TIMP) levels.

MMP-2 activation paradoxically requires the involvement of its inhibitor, TIMP-2, whereby the inactive form of the enzyme, proMMP-2, is bound by a TIMP-2/membrane type 1-MMP (MT1-MMP or MMP-14) complex at the cell membrane.²⁷ The resultant complex presents it to a second molecule of MT1-MMP on the cell surface for activation. Given the involvement of TIMP-2 in both MMP activation and regulation of active MMPs, scleral TIMP-2 must be regarded as an important regulator of scleral matrix turnover. Given this TIMP-2/MMP-2 interplay, regulation of scleral TIMP-2 levels is likely complex. Work by Lu et al.²⁸ has supported this, with data showing that low TIMP-2 levels promote MMP-2 activation, whereas as concentrations increase past a critical stoichiometric point, TIMP-2 begins to inhibit MMP-2 activity.²⁰

Although there is ample evidence of alterations in MMP-2 expression and activation during myopia development, there is less evidence available regarding the alteration of TIMP-2. Studies in chick suggest *TIMP-2* expression is down-regulated in the fibrous scleral layer of the myopic eye.²⁶ Furthermore, altered TIMP levels have been reported during increased or slowed eye growth in a number of animal model and human studies.^{15,16,19,29,30}

If reduced TIMP-2 levels are critical in controlling MMP-2 activation, scleral matrix turnover, and eye size, increasing expression should inhibit myopia development. However, work in the chick showed that administration of exogenous TIMP-2 reversed accelerated collagen degradation, but had little effect on the rate of myopia development.¹⁸ It was suggested that TIMP-2 was unable to significantly reduce myopia development in the chick due to the relatively small impact of the fibrous scleral layer on avian scleral biomechanics. It is therefore hypothesized that, assuming exogenously delivered TIMP-2 levels can exceed the stoichiometric levels necessary for maximal MMP-2 activation in the sclera, TIMP-2 supplementation during myopia development should both reduce collagen degradation and slow myopia development in mammals, which lack the rigid cartilaginous scleral layer of birds.¹⁸ In support of this, a recent study showed that reduced *MMP-2* levels and increased *TIMP-2* levels accompanied the pirenzepine-induced inhibition of myopia in the guinea pig.³⁰ Despite this, there are also contradictory findings such as those of Leung et al.³¹ who, using a gene pooling approach, showed that *TIMP-2* was not associated with high myopia.

The current study investigated three important factors to test the hypothesis that *TIMP-2* expression is altered in mammalian myopia and subsequent supplementation will slow myopia development. First, changes in endogenous scleral *TIMP* mRNA expression patterns were investigated in the tree shrew model of myopia, during both the early and intermediate stages of myopia induction. Second, the efficacy of recombinant human TIMP-2 in inhibiting the activation and activity of tree shrew MMP-2 was demonstrated in vitro. Finally, the capacity of this recombinant TIMP-2 to inhibit scleral collagen degradation during myopia induction, and the development of myopia itself, was assessed in vivo.

MATERIALS AND METHODS

Experimental Animals

Maternally reared tree shrew pups (*Tupaia belangeri*) were kept with the mother until procedures commenced 15 days after eye opening. This time point has been shown to be at the start of the susceptible period for myopia development.³²

During the experiment, each animal was placed in its own cage with food and water freely available, and the lighting of the room was set on a 14/10-hour light/dark cycle. The average illumination level at the cage floor was approximately 250 lux. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals were divided into three groups, and myopia was monocularly induced for 1 ($n = 7$), 5 ($n = 7$), or 12 ($n = 12$) days by translucent diffuser wear (form deprivation). Animals undergoing myopia induction had a pedestal fixed to their head to allow the attachment of a goggle frame for holding the translucent diffuser, as previously described.³³ Animals wore the goggle with a 12.5-mm-diameter translucent diffuser fixed with cyanoacrylate adhesive to one side of the goggle frame. The untreated eye served as a within-animal control, and left or right eye treatments were allocated randomly within each group.

Ocular Biometry

Ocular measures were collected prior to and at the end of the myopia induction period. Final measurements were collected with tree shrews anaesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg), and body temperature was maintained on the heating pad. Tropicamide (1%) was used to dilate the pupil to facilitate refractive assessment and ultrasound measurement of the ocular components. Retinoscopy was performed by two experienced observers, using a streak retinoscope and full aperture trial lenses, along vertical and horizontal meridians and to an accuracy of ± 0.50 D. Ultrasonography was carried out using a 10-MHz A-scan probe driven by a pulser-receiver (5703; Panametrics, Waltham, MA, USA), and data acquisition was controlled by a PC running a custom-designed LabView program (National Instruments, Austin, TX, USA). The probe contacted the eye through a saline interface and was positioned with an XYZ micro-manipulator. Data collected consisted of six separate measurements, each the average of 20 incoming waveforms. Data were converted from time to distance and then analyzed with the LabView software.

Dissection and Collection of Tissue

At the end of the myopia induction period, animals were administered ketamine (90 mg/kg) and xylazine (10 mg/kg) before an overdose of sodium pentobarbital (150 mg/kg) was used to induce a terminal anesthesia, and the eyes were enucleated. Tissue collection was performed, as previously described, with a 7-mm posterior scleral punch taken using a surgical trephine.³⁴ The optic nerve head was removed and discarded using a 1.5-mm trephine, and the sclera were wiped clean with a PBS-moistened cotton bud, before being snap frozen in liquid nitrogen. All tissues were stored at -80°C prior to RNA extraction.

In Vivo Expression of TIMP-2 During Myopia Development

RNA Extraction and Reverse Transcription. Scleral tissues, from animals with 1 and 5 days of induced myopia, were homogenized in a freezer mill, and total RNA was extracted from the scleral samples via a phenol/chloroform extraction method, as previously described.¹¹ Samples were treated with DNase I (Promega, Madison, WI, USA) to digest any residual genomic DNA. Purified total RNA was quantified by spectrophotometry at 260 and 280 nm to obtain an estimate of RNA concentration and purity. Total RNA (500 ng) from each sample was reverse transcribed into cDNA using 1 μM oligo dT₁₅ primer (Promega), 1 mM dNTP, 4 μL (5 \times) reaction buffer (Promega), 0.5 μL RNasin (Promega; 50 U/ μL), and 1 μL

Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). The reaction was carried out at 42°C for 1 hour followed by heating for 2 minutes at 94°C to terminate the reaction. The 20- μ L reaction was then diluted to 100 μ L with DNase/RNase-free water and stored at -20°C.

Primer Design. Primer pairs were designed against *TIMP-1*, *TIMP-2*, *MMP-2*, *MT1-MMP*, and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). *TIMP-2*, *MMP-2*, and *MT1-MMP* were investigated as key components of the MMP-2 activation complex. *TIMP-1* was investigated as a further key regulator of active MMP-2. Available sequence information for the genes of interest was first obtained from the sequence databases, specifically for mammalian species. A sequence alignment for all available sequences for a gene was carried out using the web-based program ClustalW, and areas of complete homology between species were identified. Primers were designed to target these regions of cross-species homology. The identity of all subsequent primer-generated products was confirmed through sequencing and BLAST comparisons within the databases. These specific products were also purified for use as standards in quantitative PCRs. Primer pairs used are detailed in Table 1.

Quantitative Real-Time PCR. The PCR was carried out using the LightCycler real-time PCR instrument (Roche Applied Science, Penzberg, Germany) and the fluorescent DNA-binding dye, SYBR green I. Reactions were optimized, and melting peaks were monitored so that the amplification of nonspecific product was minimized, and any nonspecific product that was amplified was not included in the quantification process. The PCR assays were conducted in triplicate, to enhance the accuracy of estimates and in accordance with the high-resolution quantitative PCR methodology previously validated and reported by this laboratory.^{11,35,36} Sample cDNA templates or dilutions of gene-specific cDNA standards (4 μ L) were mixed with primers (0.5 μ M) and the FastStart DNA Master SYBR Green I kit (Roche Applied Science). Additional magnesium chloride was added as determined through optimization of a given primer pair.

In Vitro Inhibition of MMP-2 Activation and Activity

Primary Culture of Fibroblasts. The capacity of recombinant TIMP-2 to inhibit tree shrew MMP-2 was evaluated in vitro in tree shrew skin fibroblasts. The recombinant human TIMP-2 had a predicted amino acid identity of at least 95% with the tree shrew TIMP-2 (BlastP; <http://www.treeshrewdb.org/>, in the public domain). Fibroblasts were collected from tree shrew skin, obtained from 30-day-old tree shrews under deep anesthesia before the animals were euthanized with sodium pentobarbital. The comparison of gene characteristics from the scleral and skin fibroblasts is shown in Results. To obtain skin tissue, the area around the flank of the animal was shaved with an electric razor and swabbed with 70% ethanol. A small incision was then made in the dermal layers, the incision was enlarged, and an area of skin was removed. Extraneous tissue was removed before the sample was allowed to attach to the base of a 35-mm culture dish (Nunc, Roskilde, Denmark), and cells were grown out of the sample in Dulbecco's modified Eagle's medium (DMEM, Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 25 mM HEPES (Sigma-Aldrich Corp., St. Louis, MO, USA), penicillin-streptomycin (100 U/mL; Invitrogen), and 10% fetal bovine serum (JRH, Melbourne, Australia) while kept at 37°C in a humidified incubator (5% CO₂ and 95% air). The isolated cells were then passaged and grown in primary culture dishes until they were ~85% confluent. Cells were then passaged into larger vessels to allow continued growth until sufficient numbers were available to carry out

TABLE 1. Tree Shrew-Specific Primers

Primer Pair	Sequence	Product Length, bp
TIMP-1	F: 5' ACC TGG TCA TCA GAG CCA AG 3' R: 5' CAC TCT CCC AAT TTG CAA GG 3'	379
TIMP-2	F: 5' ACG TAG TGA TCA GGG CCA AG 3' R: 5' GTG ACC CAG TAA ATC CAG AG 3'	413
MMP-2	F: 5' TGG ATG ATG CCT TTG CTC 3' R: 5' GGG TAT CCA TCT CCA TGC TC 3'	129
MT1-MMP	F: 5' CAT CTG TGA CGG GAA CTT TGA C 3'	320
HPRT	R: 5' TGG GCA TCC AGA AGA GAG C 3' F: 5' GGA GGC CAT CAC ATT GTA GC 3' R: 5' CCT GAC CAA GGA AAG CAA AG 3'	282

Tree shrew-specific oligonucleotide primers were designed against areas of high cross-species identity. Specific product size is indicated. F, forward; R, reverse.

experiments. All experiments were conducted on cells between passages 2 and 7, as this was found to be the active growth phase for the cells (data not shown).

Confirmation of Cell Expression Patterns. PCR was carried out on RNA extracted from fibroblast cells after 2, 4 and 6 passages, to ensure these cells produced *MMP-2*, *TIMP-2*, and *MT1-MMP*, the major components of the activation complex, and *TIMP-1*, another key regulator of MMP-2 activity. Scleral cDNA was used as a positive control for expression of these genes. Briefly, cells were detached, with trypsin, and RNA isolated using Nucleospin RNA II columns (Macherey-Nagel, Bethlehem, PA, USA) as per the manufacturer's instructions. Contaminating genomic DNA was removed by incubation on the column with a DNase I-containing buffer. Total RNA was eluted from the column into 100 μ L RNase-free water, quantified, checked for purity using a spectrophotometer, and stored at -80°C. Reverse transcription was carried out using the protocol described earlier. The detection of gene-specific cDNA was carried out using primers specific for tree shrew *TIMP-1*, *TIMP-2*, *MMP-2*, and *MT1-MMP*, designed and validated as described earlier, and detailed in Table 1. The primer sequence used for *MMP-2* in this particular aspect of the current study was as follows: F, 5' GAC GAT ATC AAG GGC ATT CAA GA 3' and R, 5' TTC TTG CTC CAG TTA AAA GCA GC 3', generating a product length of 425 bp.³⁷ PCR reactions were carried out using *Taq* DNA polymerase (Promega) on a Progene block cyler (initial 2-minute melting step at 94°C followed by 40 cycles of 94°C for 2 minutes, an annealing step of 1 minute at 55°C, and a 1-minute extension step at 72°C), 0.5 mM dNTPs, (10 \times) thermostable buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton-X, Promega), and optimized MgCl₂ concentrations. PCR product from each primer set was mixed with 5 μ L DNA loading dye, and the sample was loaded into a 2% agarose gel in (0.5 \times) TBE and run. The gel was photographed using a UV light box.

TIMP-2 Inhibition of MMP-2 Activation and Activity In Vitro. The efficacy of recombinant TIMP-2 in preventing the activation of tree shrew MMP-2 in culture was assessed using the tree shrew fibroblast culture model and gelatin zymography. Concanavalin A (con-A), a lectin that promotes MMP-2 activation via the MT1-MMP/TIMP-2 pathway, was used to activate latent MMP-2 (0 to 20 μ g/mL), and the dose-dependent capacity of recombinant TIMP-2 to prevent this activation was assessed. Cells were incubated for 24 hours in serum-free medium containing both 20 μ g/mL con-A and recombinant human TIMP-2 (Sigma-Aldrich Corp.) at concentrations between 0 and 20 nM. The medium was then collected and centrifuged, and amounts from each sample were loaded onto gelatin zymograms. The

zymographic characteristics of MMP-2 (latent, intermediate, and active forms) from tree shrew were confirmed and reported in a previous publication from the laboratory, and the same methodology was followed in the current study.²³ The amount of latent and active MMP-2 activity in each lane of the gel was quantified using a light box and digital photography, and then band intensity was analyzed using Kodak Digital Science software (Version 3.0.0; Rochester, NY, USA).

The capacity of recombinant TIMP-2 to inhibit tree shrew MMP-2 activity was assessed using a gelatinase assay kit (cat no: ECM700; Chemicon, Temecula, CA, USA). Briefly, cells were incubated in serum-free medium for 24 hours before the conditioned medium was collected and centrifuged to remove any debris. Latent MMP-2 in the conditioned medium was activated using p-aminophenylmercuric acetate (APMA), an organomercurial that cleaves the pro-peptide region of latent MMP-2, thus activating it through a non-MT1-MMP pathway. APMA (10 mM) in 0.1 M NaOH was diluted in TTC buffer (50 mM Tris-HCL, pH 7.5, 1 mM CaCl₂, 0.05% Triton X-100), mixed 1:9 with the conditioned medium, and incubated for 2 hours at 37°C. The activated, conditioned medium was then assayed in accordance with the manufacturer's instructions for the gelatinase assay kit. Biotin-labeled gelatin was incubated with the APMA-activated conditioned medium and 0.1 to 50 nM recombinant TIMP-2 at 37°C for 3 hours, to allow the active MMP-2 to digest the labeled gelatin. Samples were then transferred to a 96-well biotin-binding plate, washed, and detected via incubation with a streptavidin-enzyme conjugate. The undigested product was quantified on a plate reader at 405 nm.

In Vivo TIMP-2 Inhibition of Scleral Collagen Degradation and Myopia Development

In Vivo Collagen Labeling and Subconjunctival TIMP-2 Delivery. Animals ($n = 12$) were injected intraperitoneally with a radio-labeled collagen precursor (300 μ Ci [3.5 mM] L-[2,3,4,5-³H]-Proline; PerkinElmer, Waltham, MA, USA) 24 hours prior to the induction of myopia to label all new scleral collagen synthesized over the 24 hours prior to the application of the diffuser. The following day, animals were placed in one of two groups: one to receive daily subconjunctival injections of recombinant TIMP-2 into the treated eye and the other daily injections of vehicle ($n = 6$ each group). At the same time, the animal received an intraperitoneal injection of nonlabeled proline (2.3 M L-proline; Sigma-Aldrich Corp.), to dilute tissue pools of the radio-labeled proline and limit any further labeling of newly synthesized collagen during myopia induction.

Over a 12-day period, each tree shrew was weighed, anesthetized (2.5% isoflurane in oxygen), and received recombinant human TIMP-2 (125 nM in vehicle) or vehicle (1.5% w/v carboxymethyl cellulose in 0.9% saline; Sigma-Aldrich Corp.) daily. TIMP-2 or vehicle was injected into the superior (45 μ L) and inferior (45 μ L) subconjunctival space (i.e., 90 μ L each day) to form a visible bleb. The selected dose of TIMP-2 was based on the in vitro inhibition data for TIMP-2 inhibition of MMP-2 (see Results) and estimated drug distribution to the posterior scleral tissue. Previous publications from this laboratory using the chick model allowed us to estimate that a dose of approximately 0.28 pmol TIMP-2 reached a 7-mm button of the posterior polar sclera,¹⁸ which equated to a concentration of approximately 73 nM, or approximately 3.5 to 7 times the IC₅₀ for inhibition of MMP-2 activation and 35 times the IC₅₀ for the inhibition MMP-2 activity.

³H-Proline and Hydroxyproline Assays. Following the collection of ocular biometric data, an overdose of sodium pentobarbital (150 mg/kg) was administered, and a 7-mm posterior scleral button was isolated using the methodology

described above. All scleral samples were assayed for collagen degradation. The procedures for ³H-Proline assay and hydroxyproline assay were carried out exactly as detailed in previously published studies from this laboratory.^{11,18} Briefly, all samples were homogenized in sterile water and then suspended in pepsin (2 mg/mL; Sigma-Aldrich Corp.) and 0.5 M acetic acid. Samples were digested for 48 hours at 4°C with gentle shaking. Filters of wells (Multiscreen 0.65- μ m pore size Durapore membrane; Millipore, Billerica, MA, USA) were wetted with 100 μ L 25% trichloroacetic acid (TCA) before replicate samples (100 μ L) were loaded. Another 100 μ L 50% TCA was added, and the plates were incubated at 4°C with gentle shaking for 1 hour. The plate was then drained using a manifold system (Millipore) connected to a vacuum source and washed three times using 300 μ L 10% TCA to remove unincorporated label. The dried filters were punched out from the plate and placed in scintillation vials filled with 500 μ L 4 M guanidine HCL in 33% isopropanol. The vials were incubated overnight at room temperature with shaking. Ten milliliters liquid scintillation fluid (Cytoscint ES; Fisher Scientific, Pittsburgh, PA, USA) was added to each vial and analyzed (Winspectral 1414; Wallac Turku, Finland).

All radio-labeled collagen data were normalized to the hydroxyproline content of the samples. Standards of hydroxyproline were first prepared by dissolving trans-4-hydroxy-L-proline (Sigma-Aldrich Corp.) in dH₂O (0.5 to 8 μ g/40 μ L). Then 10 μ L 10 M NaOH was added to 40 μ L of each sample and standard. The tissue samples and standards were then hydrolyzed by autoclaving at 120°C for 20 minutes; 450 μ L 0.065 M chloramine-T reagent in 50% n-propanol and acetate-citrate buffer (pH 6.5; Sigma-Aldrich Corp.) was added to each sample and standard after cooling. After the reagent was mixed gently, the oxidation reaction was allowed to proceed for 25 minutes at room temperature. Then, 500 μ L 1 M Ehrlich's reagent (Sigma-Aldrich Corp.) was added to each sample and standard and mixed gently. Color changes in each sample and standard were allowed to develop in a water bath at 65°C for 20 minutes. Finally, 900 μ L of each sample and standard were transferred to a 1-mL cuvette (Polystyrene, Sarstedt, Germany), and the color density was quantified at 550 nm using a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan). The hydroxyproline content of each sample was determined from the standard curve.

Data Analysis

All data were processed in Microsoft Excel spreadsheets and analyzed with a statistical package (GraphPad Prism, La Jolla, CA, USA). All group data were expressed as mean \pm SEM. The amount of active MMP-2 was expressed as a ratio of total MMP-2 present in the lane. This value was normalized to the control (no inhibitor) ratio, which was assigned an arbitrary value of 1.0. Data were transferred to GraphPad Prism and fit with a sigmoidal dose-response curve and the IC₅₀ calculated. The scleral radio-labeled collagen data were normalized to hydroxyproline content. TIMP-2 expression data and the scleral radio-labeled collagen data were expressed as the percentage difference between treated and control eyes (% Difference [T - C]/C). Ocular biometric data were expressed as the difference between treated and contralateral control eyes or absolute values. Data were analyzed through either one-sample or two-sample *t*-test.

RESULTS

Changes of TIMP-1, TIMP-2, MMP-2, and MT1-MMP Expression in Myopia

After 1 and 5 days of form deprivation, relative changes of TIMP and MMP mRNA expression were investigated. Animals

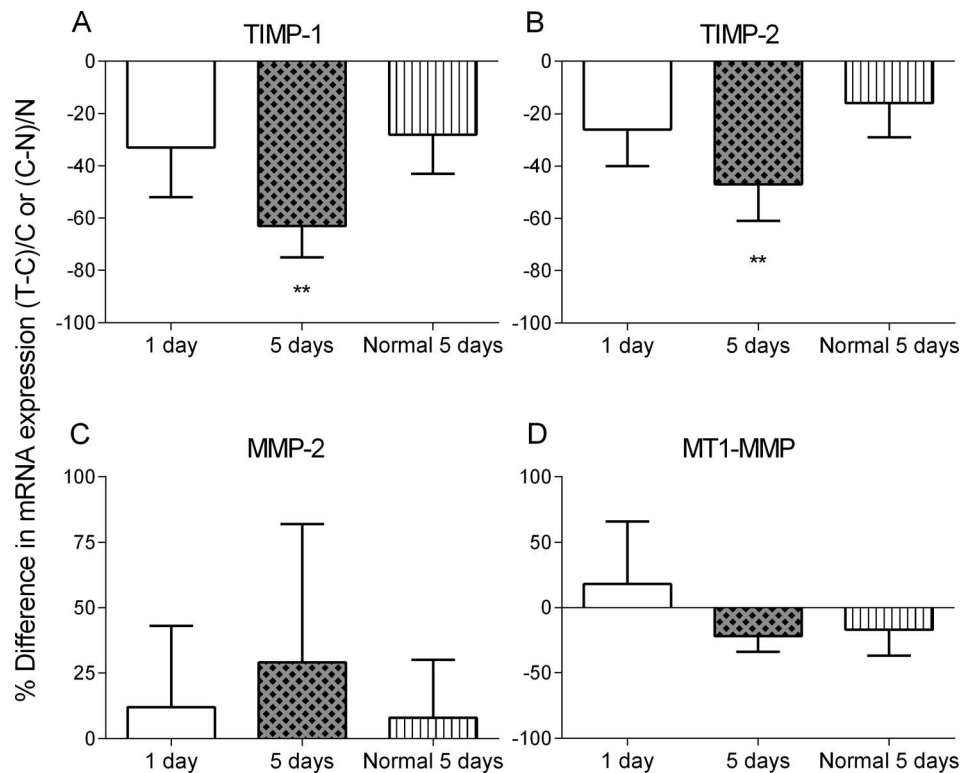


FIGURE 1. Relative differences in expression of *TIMP-1*, *TIMP-2*, *MMP-2*, and *MT1-MMP* mRNA in the tree shrew sclera after 1 and 5 days of form deprivation. A significant down-regulation in *TIMP-1* and *TIMP-2* expression was noted after 5 days of monocular form deprivation. Normal 5-day data represent the relative difference between contralateral control eyes of 5-day form-deprived eyes and normal eyes (average of right and left eyes) of age-matched untreated animals. Data were normalized to *HPRT* expression and presented as percentage mean expression difference: (treated – control eyes)/control eyes or (control – normal eyes)/normal eyes ± SEM. $n = 7$ each group, ** $P < 0.01$.

in the 1 day group underwent no further ocular measures after myopia induction because it had been previously demonstrated that significant changes in refractive parameters were not yet apparent at this stage.³⁸ Following 5 days of myopia induction, an average of -7.8 ± 0.7 D of relative myopia was induced.

Quantitative PCR data showed decreased *TIMP-1* and *TIMP-2* mRNA expression in eyes with relative myopia (Figs. 1A, 1B, respectively). This difference between treated and control eyes increased in relative magnitude between the shorter and longer period of form deprivation, with a significant decrease noted after 5 days of form deprivation (*TIMP-1*, 1 day: $-33 \pm 18\%$, $P = 0.14$, 5 days: $-63 \pm 12\%$, $P < 0.01$; *TIMP-2*, 1 day: $-26 \pm 14\%$, $P = 0.12$, 5 days: $-47 \pm 14\%$, $P < 0.01$). The expression of *MMP-2* was not different between treated and control eyes at any time point examined ($P > 0.05$), although there was a general trend of increased expression in the treated eyes (Fig. 1C). For *MT1-MMP* (Fig. 1D), no significant difference in expression between treated and control eyes was found after 1 or 5 days of monocular deprivation ($P > 0.05$). Gene expression data from all contralateral control eyes of 5-day form-deprived eyes were not significantly different from normal eyes of age-matched animals ($P > 0.05$; Figs. 1A–1D).

In Vitro Inhibition of MMP-2 Activation and Activity

Before examining the capacity of recombinant human TIMP-2 to inhibit the activation and activity of tree shrew MMP-2, verification of the tree shrew skin fibroblast culture model was carried out. As expected, Figure 2 illustrates that PCR products of the appropriate size to represent *TIMP-1*, *TIMP-2*, *MMP-2*,

and *MT1-MMP* mRNAs are expressed both in tree shrew skin fibroblasts and in scleral cDNA, the positive control. The expression of each of these genes persisted through passages 2, 4, and 6, thus verifying the model's validity for the subsequent experiments.

To determine whether recombinant human TIMP-2 could be used to modulate tree shrew MMP-2 activation, we used an in vitro culture-based system. Tree shrew skin fibroblasts were treated with con-A and TIMP-2 to influence activation of endogenous MMP-2. As can be seen in Figure 3A, increasing concentrations of con-A (0 to 20 $\mu\text{g/mL}$) resulted in increasing levels of the active form of MMP-2, allowing us to approximate the dose-response characteristics. When tree shrew skin fibroblasts were incubated with con-A (20 $\mu\text{g/mL}$) and increasing concentrations of TIMP-2, MMP-2 activation was inhibited in a dose-dependent manner (Figs. 3B, 3C). Partial inhibition of activation was evident at a concentration of 5 nM TIMP-2, whereas the IC_{50} was estimated as 10 to 20 nM from the dose-response curve (Fig. 3C).

The capacity of recombinant TIMP-2 to modulate active tree shrew MMP-2 was also tested using cultured cell medium from the skin fibroblast model. After treatment with APMA, to activate MMP-2, the medium was incubated with TIMP-2 in the presence of a biotin-labeled gelatin substrate, to determine MMP-2 activity levels. As can be observed in Figure 4, recombinant human TIMP-2 was able to inhibit active MMP-2 in this system, with an estimated IC_{50} of 2 nM.

TIMP-2 Effect on Myopia Development

To determine whether supplementation of TIMP-2 can alter myopia development, recombinant human TIMP-2 was deliv-

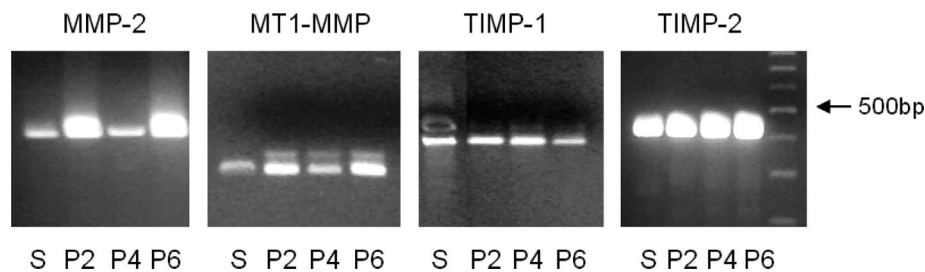


FIGURE 2. Both tree shrew scleral tissue and skin fibroblasts express mRNA for *MMP-2*, *MT1-MMP*, *TIMP-1*, and *TIMP-2*. PCR products were generated using gene-specific primers and visualized on a 2% agarose gel stained with ethidium bromide. S, cDNA from scleral tissue; P2, P4, P6, cDNA from skin fibroblast cells at passages 2, 4, and 6 respectively.

ered to tree shrew eyes in vivo. Prior to myopia induction, there were no differences in refraction between the treated and control eyes in the both groups investigated (data not shown). A significant amount of relative myopia, vitreous chamber elongation, and increased axial length was observed after 12 days of monocular deprivation of pattern vision in the vehicle-treated group (Table 2). In contrast, the TIMP-2-treated group exhibited reduced levels of myopia, decreased vitreous chamber elongation, and shorter eyes (Table 2). Although the vehicle control group developed -10.0 ± 0.6 D of relative myopia

during the deprivation period, the delivery of exogenous TIMP-2 effectively halved the amount of relative myopia produced during the deprivation period (Fig. 5A; -4.9 ± 0.5 D, $P < 0.01$). The relative myopia that developed was largely associated with the relative differences in vitreous chamber depth (Fig. 5B) and was reflected in an increased axial length (Fig. 5C). There were no significant differences in measurements obtained for anterior chamber depth or lens thickness (data not shown). Treatment with TIMP-2 produced significant differences in vitreous chamber elongation (Fig. 5B; vehicle

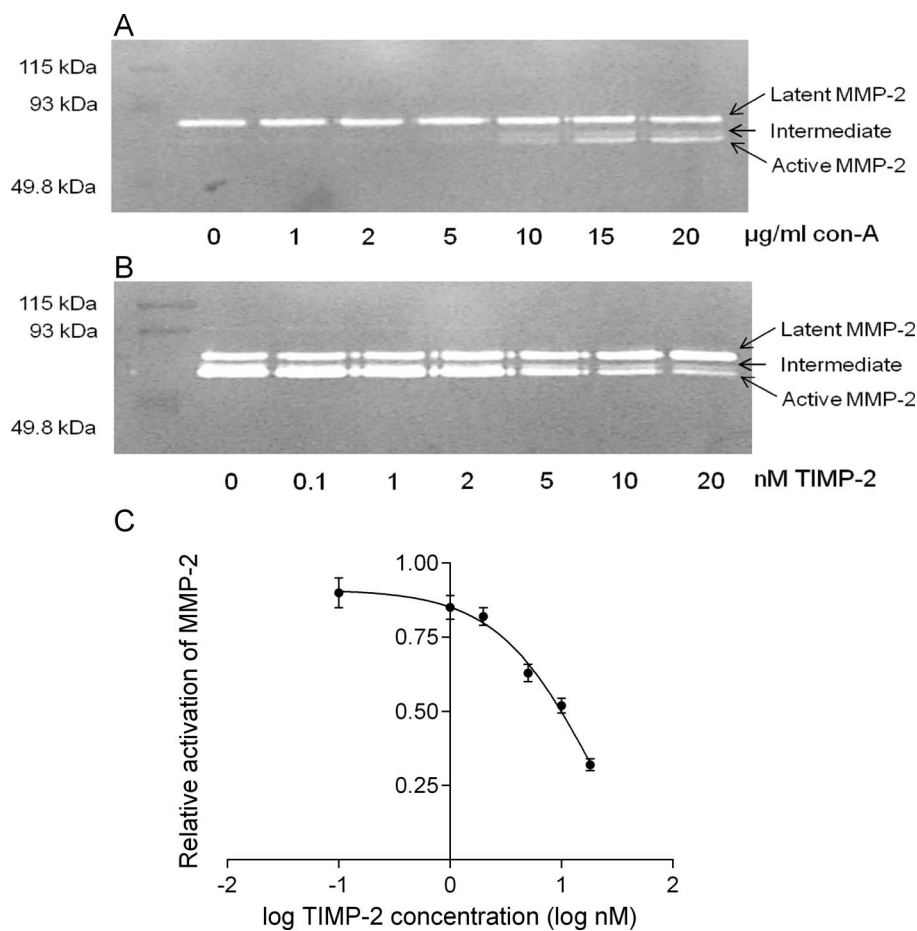


FIGURE 3. Con-A promotes MMP-2 activation and human TIMP-2 prevents the con-A-induced activation of tree shrew MMP-2 in a dose-dependent fashion. (A) Gelatin zymogram showed the effect of con-A at concentrations of 0 to 20 µg/mL, incubated overnight with confluent tree shrew skin fibroblasts. Numbers in left margin refer to the molecular weight markers. (B) Gelatin zymogram showed the effect of TIMP-2 on the con-A-induced activation of MMP-2. Tree shrew skin fibroblast cells were incubated for 24 hours with 20 µg/mL con-A and 0–20 nM TIMP-2. (C) Dose-response curve showed the effect of TIMP-2 addition on MMP-2 activation. Activation was calculated as the proportion of total MMP-2 that was present in the active form. Activation was normalized to the control well, containing 20 µg/mL con-A only, and assigned a value of 1.0. Data expressed as mean \pm SEM, $n = 7$.

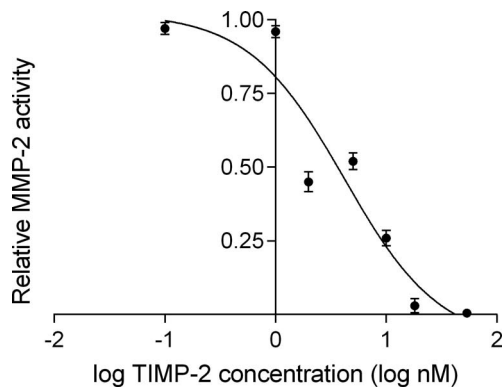


FIGURE 4. Human TIMP-2 prevents the digestion of biotin-labeled gelatin substrate by active tree shrew MMP-2. Conditioned medium from tree shrew fibroblast cells was activated with APMA and assayed using a plate-based kit for MMP-2 activity against biotin-labeled gelatin with the addition of 0 to 20 nM TIMP-2. Values were normalized such that the control was assigned a value of 1.0. Data presented as mean \pm SEM, $n = 7$.

group, 0.21 ± 0.01 mm versus TIMP-2 group, 0.13 ± 0.01 mm, $P < 0.01$) and axial length elongation (Fig. 5C; vehicle group, 0.23 ± 0.02 mm versus TIMP-2 group, 0.15 ± 0.01 mm, $P < 0.05$). These data suggest that, at the concentrations used, TIMP-2 significantly inhibited the development of myopia while not completely preventing it.

TIMP-2 Regulation of Collagen Degradation

As altered collagen turnover is a key scleral correlate of myopia development, the effect of TIMP-2 on the rate of relative scleral collagen degradation was investigated in both groups. Form-deprived eyes treated with vehicle showed a reduced level of scleral-labeled proline content (124 ± 4 decay per minute [DPM]/ μ g) from control eyes (152 ± 7 DPM/ μ g, $P < 0.01$), over the 12-day period (Fig. 6A). By contrast, there was no alteration in labeled proline content in TIMP-2-treated eyes (140 ± 13 DPM/ μ g) after 12 days of myopia induction compared with control (Fig. 6A; 141 ± 11 DPM/ μ g, $P > 0.05$). When this relative decrease in proline incorporation was expressed as collagen degradation, it was found to be significantly increased in the sclera of vehicle-treated eyes ($19 \pm 1\%$, $P < 0.01$; Fig. 6B). However, there was no significant

TABLE 2. Tree Shrew Refractive and Ocular Biometric Data

Group/Eye	Refraction, D	Vitreous Chamber Depth, mm	Axial Length, mm
Vehicle group			
Treated	-1.4 ± 0.8	3.00 ± 0.02	7.34 ± 0.03
Control	8.7 ± 0.5	2.79 ± 0.02	7.10 ± 0.03
Treated – control	-10.0 ± 0.6	0.21 ± 0.01	0.23 ± 0.02
TIMP-2 group			
Treated	4.1 ± 0.3	2.90 ± 0.03	7.26 ± 0.02
Control	9.0 ± 0.3	2.77 ± 0.03	7.11 ± 0.03
Treated – control	$-4.9 \pm 0.5^{**}$	$0.13 \pm 0.01^{**}$	$0.15 \pm 0.01^*$

Refractive data were obtained using streak retinoscopy, whereas ocular dimensions were quantified using A-scan ultrasound. Measures were taken following 12 days of monocular deprivation and treatment with either vehicle or TIMP-2. Data are expressed as the mean \pm SEM. $n = 6$ for each group. Significant difference between the two groups: $^{**}P < 0.01$ and $^*P < 0.05$.

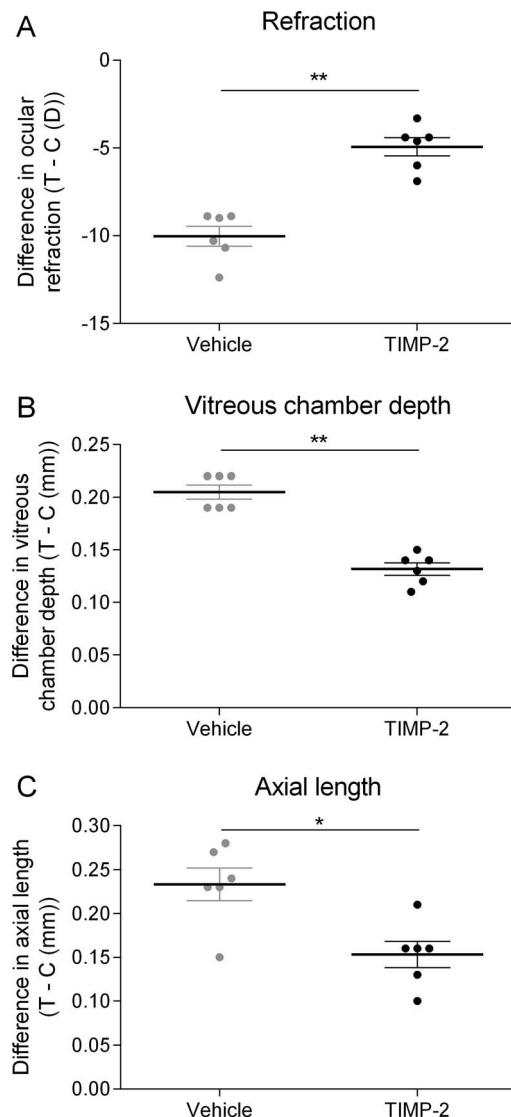


FIGURE 5. The effect of recombinant human TIMP-2 addition on myopia development. (A) A significant reduction in relative myopia was found in the TIMP-2-treated, form-deprived animals compared with the vehicle-treated group. Significant decreases in vitreous chamber depth (B) and axial length (C) were the biological correlate of refractive findings in TIMP-2-treated animals compared with vehicle-treated animals. Data presented as mean \pm SEM, $n = 6$ each group, $^{**}P < 0.01$, $^*P < 0.05$.

relative difference in scleral collagen degradation in TIMP-2-treated eyes compared with contralateral control eyes ($2 \pm 3\%$; Fig. 6B). This suggests that, during myopia development, the replenishment of TIMP-2 levels in the sclera completely inhibited the degradation of collagen that had previously been labeled with the radio-labeled precursor, despite the fact that the structural changes were only partially inhibited.

DISCUSSION

The current study contributes three important findings to the literature regarding the role of TIMP-2 in the scleral changes associated with the development of myopia in mammalian models. First, the study confirmed that there is a reduction in expression of *TIMP-2* and *TIMP-1* in the posterior sclera of mammalian eyes that are developing myopia, the change in

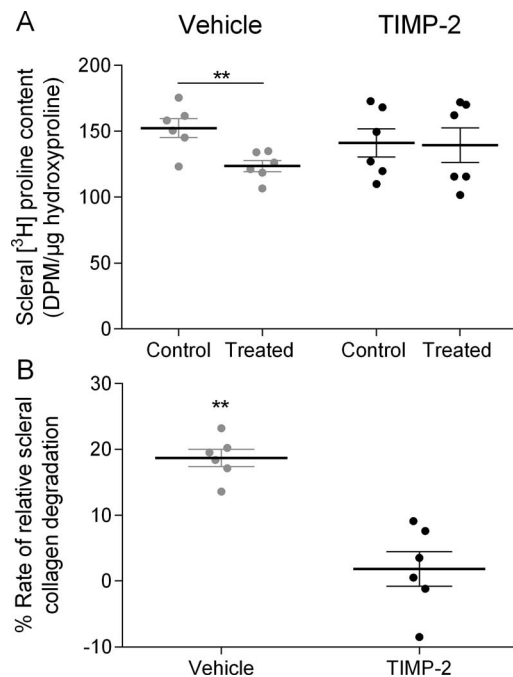


FIGURE 6. The effect of recombinant human TIMP-2 on scleral collagen degradation. (A) ³H-Proline levels in vehicle, TIMP-2 and contralateral control eyes. Eyes treated with vehicle showed significantly increased scleral collagen degradation, whereas no difference in collagen degradation, relative to control eyes, was found in recombinant TIMP-2-treated eyes after 12 days of myopia induction. (B) A significant relative increase in scleral collagen degradation was found in the vehicle group, but not the TIMP-2 group, indicating MMP-mediated collagen degradation had been significantly inhibited in TIMP-2-treated animals. Data expressed as mean ± SEM, $n = 6$ each group, $**P < 0.01$.

TIMP-2 being of particular interest due to its biphasic role in both activation and inhibition of MMP-2. These reductions were found to be, initially at least, time dependent, with a greater reduction after a longer period of myopia development. Second, the *in vitro* study demonstrated that tree shrew MMP-2 activation and activity can be regulated using low nanomolar levels of recombinant human TIMP-2 and that this regulation is dose dependent. Third, the study showed that subconjunctival supplementation with physiologically relevant levels of TIMP-2, to offset the reduction in scleral TIMP-2 levels caused by form deprivation, completely inhibited degradation of newly labeled scleral collagen and partially inhibited experimentally induced myopia. Collectively, these results demonstrate that myopia development in mammals is associated with decreased TIMP-2 and TIMP-1 expression, presumably contributing to increased, MMP-related, collagen degradation in the sclera, and that inhibition of this scleral matrix degradation is achievable through supplementation with MMP-inhibitors, also leading to slowed myopia progression.

A relative decrease in TIMP-2 and TIMP-1 mRNA expression was found during form deprivation-induced myopia, which increased in magnitude over time, becoming significant after 5 days of monocular deprivation. These data are supported by previous work in the guinea pig model of myopia, where scleral TIMP-2 mRNA expression was reduced by ~31% after 10 days of deprivation.³⁰ In addition, work in the fibrous sclera from the chick model of myopia also showed reduced TIMP-2 levels.²⁶ A relatively recent study on highly myopic patients showed altered MMP-2/TIMP-2 ratios in isolated vitreous samples.¹⁹ As the overall balance between MMP/TIMP expression governs the scleral remodeling that facilitates excessive ocular elonga-

tion, these contradictory data may reflect species-specific differences in the regulation of other MMPs and/or TIMPs in the human eye. For instance, alterations in other MMPs such as MT1-MMP may result in the elevated TIMP-2 levels.

In contrast to findings with TIMPs, MMP-2 gene expression was variable, and not significantly altered, as was MT1-MMP gene expression, after 1 or 5 days of myopia development. This suggests that the increased, MMP-2-dependent collagenolytic activity in eyes developing myopia is primarily a function of altered TIMP regulation of activation and activity rather than altered expression of MMPs. These findings were surprising given that previous investigators, also using the tree shrew model, found that, although TIMP-1 and TIMP-2 levels did not change, relative to control eyes, in myopia, MMP-2¹⁵ and MT1-MMP^{15,29,39} expression were up-regulated.¹⁵ Comparisons are difficult with the previous study as myopia was induced with negative lenses, rather than diffusers, and the animals came close to fully compensating for these lenses by the end of the myopia induction period. This was not the case in the current study in which animals developed nearly twice the myopia as the previous study. In addition, the previous study used whole sclera, rather than sclera from the posterior pole region, and variation between scleral regions has been previously reported.^{4,6} Finally, the previous study used ribosomal 18S as its housekeeping gene, whereas the current study used HPRT.⁴⁰

The ability of recombinant human TIMP-2 to influence the activation state of MMP-2 was assessed in this study, as was the capacity of TIMP-2 to inhibit activated MMP-2. MMP-2 was found to be expressed in the latent form by skin cells *in vitro*, as is the case with most fibroblast cell types grown in a monolayer.^{41,42} Incubation with the plant lectin con-A led to the activation of MMP-2 in tree shrew skin cells as has been reported for other fibroblast cells, including human corneal, dermal, and gingival fibroblasts.⁴²⁻⁴⁴ This activation occurred at similar con-A concentrations to those reported for corneal fibroblasts.⁴⁴ The activation mechanism of con-A is thought to be via the MT1-MMP-mediated pathway.⁴⁵ In the current study, this activation process was prevented by incubation with TIMP-2, which has a documented inhibitory effect against MT1-MMP.⁴⁶ TIMP-2 was also able to inhibit the breakdown of gelatin by active MMP-2 at a dose consistent with that previously shown to be effective in other species.¹⁸ The effective concentration was within the low nanomolar range, which is a little higher than expected, based on reported k_i values for TIMP-2 against MMP-2 of <9 pM,⁴⁷ but not entirely inconsistent with this value. The capacity of TIMP-2 to inhibit both MMP-2 activation and activity highlights its therapeutic potential in the management of myopia. Although TIMP-2 also has roles in cell proliferation regulation, aside from its interaction with MMPs,⁴⁸ these findings demonstrate it was reasonable to expect that TIMP-2 would be effective in the next stage of the current study, in both inhibiting the activation of MMP-2 in the tree shrew sclera during myopia progression and directly preventing the degradation of scleral tissue. The capacity of TIMP-1 to inhibit MMP-2 was not investigated, as it was reported to have relatively low affinity for MT1-MMP, implying a limited effect on the MMP-2 activation pathway.⁴⁹

TIMP-2 has a dual role in the regulation of MMP-2. It can activate proMMP-2, through its capacity to form complexes with membrane-bound MT1-MMP, and is also able to directly inhibit active MMP-2 and MT1-MMP.⁵⁰ These dual roles of TIMP-2 imply that TIMP-2 may both promote and inhibit the activation, and subsequent activity, of MMP-2 depending on the relative local concentrations present in the tissue. Although moderate levels of TIMP-2 are necessary for optimal activation of MMP-2, it has also been shown that higher levels of TIMP-2 retard the activation of MMP-2, presumably through inhibition of MT1-MMP via occupation of all MT1-MMP binding sites,

leaving no TIMP-2-free MT1-MMP available for the activation of MMP-2. Paradoxically, studies have also found that lower levels of TIMP-2 inhibit the activation of MMP-2 as a TIMP-2/MT1-MMP complex is necessary for the activation of MMP-2. As most of the MMP-2 present in the tree shrew sclera is in the latent form,²³ it can be hypothesized that the normal scleral levels of TIMP-2 are in the high inhibitory range. The mean, normalized expression levels of *MMP-2*, *MT1-MMP*, and *TIMP-2* across control eyes of the current study (*MMP-2*: 14145, *MT1-MMP*: 1079, *TIMP-2*: 5521; copies per copy of *HPRT*) were consistent with this prediction, suggesting that expression levels of *TIMP-2* in the sclera are around five times higher than expression levels of *MT1-MMP*, indicating that most scleral *MT1-MMP* will be bound to *TIMP-2* at the cell surface and little *TIMP-2*-free *MT1-MMP* will be available for *MMP-2* activation. It follows that any decrease in *TIMP-2*, from this steady-state level, as has been demonstrated in form-deprived eyes in the current study, would be expected to lead to an increase in *MMP-2* activity through decreasing inhibition of *MT1-MMP* activity, thus accelerating the activation of *MMP-2*, as well as decreasing the levels of direct inhibition of active *MMP-2*. Such increased levels of active *MMP-2* have been consistently documented during myopia development.^{23,26}

In vivo delivery of *TIMP-2* to the sclera of form-deprived eyes was found to both completely prevent degradation of scleral collagen and significantly inhibit axial myopia development, mainly through inhibition of vitreous chamber enlargement. It must be acknowledged that a “yoking effect,” between treated and contralateral control eyes, has been reported in other studies.⁹ However, we compared the contralateral control eye data (refraction, vitreous chamber depth, and axial length) of the vehicle and the *TIMP-2* groups and found no significant difference, suggesting that, in the current study, *TIMP-2* treatment had no yoking effect in the control eyes. Based on previously published studies,¹⁸ the dose of *TIMP-2* delivered subconjunctivally was estimated to result in a concentration of approximately 70 nM in the 7-mm button of tree shrew sclera, which is approximately 3.5 to 7 times the IC_{50} value of *MMP-2* activation. The “excess” dose of *TIMP-2* was selected to try and ensure that a high level of inhibition was ensured that was close to the maximum biological effect possible. Indeed, this resulted in nearly 90% of collagen degradation being inhibited by *TIMP-2*. This outcome exceeds the effectiveness of a similar treatment in the chick model of myopia¹⁸ and could be interpreted as demonstrating that at least 90% of collagen degradation in the sclera of myopic tree shrew eyes is facilitated by *TIMP-2*-sensitive MMPs. In addition, and unlike the findings in the chick model of myopia,¹⁸ data from this study also demonstrated that significant inhibition of scleral collagen degradation correlates with a significant inhibition of the development of myopic refractive error in the tree shrew. This finding is perhaps not surprising, given the structural differences in the sclera between birds and mammals,^{13,51} and the fact that in mammals, significant scleral thinning is associated with increased collagen degradation and is one of the major scleral characteristics of myopia.^{3,4} It follows that supplementation of *TIMP-2* to the mammalian sclera during form deprivation, to replace that lost due to the down-regulated mRNA expression, presumably prevents this thinning and therefore limits the development of myopia.

An interesting outcome of this investigation was that the near complete prevention of scleral collagen degradation (90%) did not translate into full inhibition of the relative myopia development, with nearer to 50% of the myopia inhibited only. This demonstrates that the development of myopia is not solely associated with the degradation of scleral collagen and implicates other changes within the scleral matrix as having

a contributory role in the development of myopia. Indeed, scleral collagen accumulation, which can be considered as the major determinant of scleral dry weight, and therefore, to a degree, scleral thickness, is associated with alteration in the balance between both collagen synthesis and degradation. It follows that, in addition to increased scleral collagen degradation, reduced scleral collagen synthesis had also been reported as a feature of myopia development in the tree shrew model of myopia.¹¹ Consequently, it may be argued that supplementation of the collagen production pathway might also be necessary to completely prevent the effects of form deprivation. It should also be noted that altered metabolism of scleral proteoglycans are an important feature of scleral remodeling in the myopic eye^{52,53} and that many of these mechanisms are not likely to be sensitive to *MMP*-related degradation pathways. Further testing of these other factors may help further understand the relative contributions of collagen and proteoglycan metabolism during myopia development and help develop effective interventions. Although positive outcomes were found through the in vivo delivery of *TIMP-2*, providing useful insights into myopia pathogenesis, it will be necessary to fully explore the complex biological roles of the matrix metalloproteinases and their inhibitors before using/targeting these factors in any future therapeutic strategy.

CONCLUSIONS

In summary, this study demonstrated that decreased endogenous scleral *TIMP-2* expression is a feature in the posterior sclera of a mammalian model of myopia and that this may promote accelerated scleral collagen degradation. In support of this prediction, data showed that scleral supplementation with exogenous *TIMP-2* in vivo was effective in inhibiting the rate of collagen degradation and reducing myopia progression, possibly through suppression of *MMP-2* activation and activity. These findings provide further evidence of an important role for the *TIMP*/*MMP* system in human myopia and in turn suggest directions that may lead to development of clinical treatments for controlling the ocular pathology, associated with high degrees of myopia, in the future.

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