ANIMAL MODELS

Cause and Effect Relationship between Changes in Scleral Matrix Metalloproteinase-2 Expression and Myopia Development in Mice

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Myopia is a serious sight-compromising condition in which decreases in scleral biomechanical strength are associated with protease up-regulation resulting in thinning of its collagenous framework and changes in the extracellular matrix composition. Matrix metalloproteinase (MMP)-2 is one of the known proteases mediating these alterations. To determine whether MMP-2 up-regulation precedes myopia development, the direct effects of gain and loss in *Mmp2* gene function were evaluated on refractive development and form deprivation myopia in mice. Four weeks after injecting an adeno-associated virus serotype 8 packaged *Mmp2* overexpression vector (AAV8-*Mmp2*), scleral MMP-2 up-regulation was accompanied by significant myopia in a normal visual environment. In contrast, AAV8 packaging with shRNA targeting *Mmp2* inhibited rises in MMP-2 expression induced by form deprivation by 54% and reduced myopia development by 23% compared with eyes injected with an irrelevant scrambled sequence. Because opposing changes in MMP-2 protein expression levels had corresponding effects on myopia progression, up-regulation of this protease contributes to inducing this condition. This notion of a cause-and-effect relationship between MMP-2 up-regulation and myopia development is supported by showing that form-deprived myopia development was attenuated by 27% in fibroblast-specific *Mmp2* deletion (*S100a4^{Cre}Mmp2^{f/f}*) mice relative to *Cre*-negative littermates (*Mmp2^{f/f}*). Therefore, MMP-2 is a potential drug target for inhibiting myopia progression. (*Am J Pathol* 2018, 188: 1754–1767; <https://doi.org/10.1016/j.ajpath.2018.04.011>)

Myopia is the most prevalent refractive error resulting in blurred vision, which in more severe cases can lead to retinal detachment, choroidal neovascularization, and possibly blindness.¹ Despite the availability of low-dose atropine and orthokeratology to slow myopia progression, which can be implemented to treat high-risk myopia in children, atropine has limited effectiveness to slow myopia progression. Therefore, there is an urgent need to identify alternative strategies to reverse the structural changes in the posterior sclera that occur during myopia development.² Myopia mostly develops as a consequence of excessive axial length (AL) elongation and increases in vitreous chamber depth (VCD). The changes are accompanied by declines in scleral biomechanical strength caused by

collagen fiber thinning and extracellular matrix (ECM) remodeling identified in human sclera and other experimental models.^{3–7} The decline in type I collagen accumulation in large part accounts for most of the increases in

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scleral distensibility because its dry weight is equal to approximately 90% of the scleral dry weight.^{8,9} These losses result from synthesis inhibition along with increases in collagen degradation.^{10–18} Furthermore, ECM remodeling stems from cytokine up-regulation that mediates changes in scleral cell phenotype and up-regulation of a host of different types of proteases.¹⁹ However, it is unclear whether these increases in protease expression precede myopia development progression or develop as a consequence of myopia.

Matrix metalloproteinase (MMP)-2 up-regulation contributes to scleral framework restructuring and ECM remodeling in tree shrews,^{20,21} guinea pigs,²² and chicks^{19,23–25} during myopia, whereas during reversal of this process declines occur in scleral MMP-2 expression. In addition to promoting collagen degradation, MMP-2 up-regulation contributes to mediating collagen remodeling by inhibiting collagen synthesis during fibrosis and tumor formation in other tissues.^{26–29} Furthermore, increases in the MMP-2 human aqueous levels are strongly correlated with AL elongation.^{30,31}

Another factor that affects the ECM remodeling process is the balance between the levels of MMPs and their natural endogenous inhibitors, that is, tissue inhibitors of matrix metalloproteinases.^{21,22,24,32} The role of changes in tissue inhibitors of matrix metalloproteinase-2 expression in ECM remodeling is species specific because this inhibitor suppressed the activities of scleral MMPs and collagen degradation as well as myopia development in tree shrews,³³ whereas it failed to inhibit myopia development in chicks.³⁴ Even though MMP-2 involvement in myopia has been extensively characterized, heretofore genetically modified animals have not been used to determine whether there is a cause-and-effect relationship between MMP-2 up-regulation and myopia in mice. To address this question, we contrasted the individual effects of either gain or loss of *Mmp2* gene function on refractive development and myopia progression.

Here, the time-dependent effects of alterations in MMP-2 expression on eye growth in a normal vision environment were compared with those occurring in a vision-obstructed environment to induce myopia. To induce myopia, a well-described mouse myopia model was used in which visual acuity was disrupted by outfitting mice with translucent diffusers that degrade visual acuity. With this form deprivation (FD) model, parallel changes in type I collagen expression levels were used as an index for evaluating variations in scleral biomechanical strength that are associated with loss and gain of MMP-2 function. Furthermore, the role of MMP-2 up-regulation in initiating myopia was determined by comparing FD myopia (FDM) development in a fibroblast-specific *Mmp2* deletion mouse with that in a *Mmp2* gene-modified mouse. The results show that FD induces scleral MMP-2 up-regulation which confirms that increases in MMP-2 expression contribute to initiating myopia development in mice.

Materials and Methods

Animals

Animal work in this study was approved by the Animal Care and Ethics Committee at Wenzhou Medical College, Wenzhou, China. All experiments were conducted in adherence with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. All of the mice were bred in the animal breeding unit at Wenzhou Medical College (Wenzhou, China). They were raised with a 12-hour light/12-hour dark cycle, and the luminance was approximately 100 to 200 lux.

Generation of Fibroblast-Specific *Mmp2*-Deficient Mice

Because this mutant was not available, the *Mmp2*^{fl/fl} mice were first developed on a C57BL/6J background in the Model Animal Research Center at Nanjing University (Nanjing, China). Two *loxP* sites were inserted flanking exon 2 of the *Mmp2* gene and bred to mice expressing the Cre recombinase enzyme, which resulted in offspring with exon 2 deleted in the Cre-expressing tissue. Frame-shift caused by lack of exon 2 deletes an amino acid sequence causing MMP-2 dysfunction.³⁵ *Mmp2*^{fl/fl} mice were crossed with *S100a4-Cre* mice (stock number 012641; The Jackson Laboratory, Bar Harbor, ME) to generate fibroblast-specific *Mmp2* deletion (*S100a4*^{cre}*Mmp2*^{fl/fl}) mice, and the Cre negative littermate (*Mmp2*^{fl/fl}) mice served as the control. Figure 1, A–E, illustrates the detailed strategy used for obtaining the indicated targeted *Mmp2* gene fibroblast deletion. PCR analysis of tail-extracted genomic DNA confirmed the predicted identity of the mice.

To determine whether there is Cre recombinase expression in S100a4-expressing scleral cells, the *S100a4*^{cre} mice were crossed with the *ROSA*^{mT/mG} mice (stock number 007676; The Jackson Laboratory) to generate *S100a4*^{cre}*ROSA*^{mT/mG} mice and the Cre-negative littermate (*ROSA*^{mT/mG}) mice. Cell membrane-localized tdTomato fluorescence (red) expression is widespread in cells in the *ROSA*^{mT/mG} mice. Cre recombinase-expressing cells are evident where the red fluorescence was replaced by cell membrane-localized enhanced green fluorescent protein (GFP; green) expression in the *S100a4*^{cre}*ROSA*^{mT/mG} mice.

Induction of FDM and Ocular Biometric Measurements in Mice

Monocular FD was induced by occluding the right eyes with translucent diffusers³⁶ (Supplemental Figure S1). The form-deprived eyes are designated as FD-T, whereas the untreated left fellow eyes were designated as FD-F. Ocular refraction was measured in a dark room by using an eccentric infrared photorefractor. The ocular biometric variables, AL, VCD, corneal radius curvature (CRC), and lens thickness (LT), were measured by using a custom-made optical coherence tomograph.³⁶

Figure 1 Strategy for targeted fibroblast *Mmp2* gene deletion. **A:** Targeting of mice *Mmp2* locus. *LoxP*-Neo cassette was inserted into the region between exons 1 and 2, and a single *loxP* site was inserted in the region between exons 2 and 3. After crossing with *S100a4-Cre* mice, exon 2 of *Mmp2* was deleted. Agarose gel electrophoresis was performed to verify the PCR products amplified from genomic DNA of *S100a4^{cre}Mmp2^{fl/fl}* (lane 1 to 3) and *Mmp2^{fl/fl}* (lane 4 to 6) mice. **B–D:** PCR identification of 5' (**B**) and 3' (**C**) *loxP* sites and Cre recombinase sequence (**D**) predicted 568-, 524-, and 490-bp products, respectively, using the primers listed in Table 1. **E:** The primer pair for null listed in Table 1 amplified a fragment of approximately 615 bp in *S100a4^{cre}Mmp2^{fl/fl}* mice but failed to generate any products in the *Mmp2^{fl/fl}* mice. FRT, flippase recognition target; UTR, untranslated region.

Construction of AAV8-*Mmp2* Overexpression Vector

At Biowit Technologies (Shenzhen, China), the cDNA sequence (1989 bp) of mouse *Mmp2* (NM_008610.3) was cloned into the adeno-associated virus serotype 8 (AAV8) plasmid that yielded AAV8-*Mmp2*. After sequence verification, amplicons were packaged by using a three-plasmid transient transfection of human embryonic kidney 293T cells (ATCC, Manassas, VA). The AAV8 supernatant was collected 48 hours after transfection, and the virus was released from the human embryonic kidney 293T cells through three consecutive freeze–thaw cycles (–80°C and 37°C). The empty AAV8 vector was also packaged, yielding the AAV8-Vector. After purifying and concentrating by ultrafiltration, the titrated virus yields ranged from 10^{12} to 10^{13} genomic copies/mL.

Construction of AAV8-shRNA Expression Vector Targeting *Mmp2*

Screening of shRNA sequences targeting mouse *Mmp2* gene was performed in NIH-3T3 cells (ATCC). The sequences for the shRNA targeting were 5'-GGGA-GATTCTCACTTTGATGA-3'. The shRNA-*Mmp2* and the

scrambled control shRNA sequences were respectively ligated into AAV8-shRNA shuttle vector to construct AAV8-shRNA-*Mmp2* and AAV8-shRNA-scramble (labeled as shRNA-*Mmp2* and shRNA-scramble) for AAV8 packaging. They were packaged according to the procedure described in “Construction of AAV8-*Mmp2* Overexpression Vector,” providing titrated yields of virus that ranged from 10^{12} to 10^{13} genomic copies/mL.

Method for AAV8 Injection

Injection was performed under a Zeiss (Gottingen, Germany) operating microscope in the right eye of each mouse. Mice were narcotized with a mixture of ketamine (72 mg/kg) and xylazine (4 mg/kg) by i.p. injection. Hydroxymethyl cellulose (2.5%) was applied to avoid desiccation before surgery. Posterior Tenon's capsule was entered along the eyeball radian from 14 to 18 mm away from the corneal limbus and eyeball equatorial axis, followed by delivery of 5 μ L of virus. Eyes were covered with 0.2% polyacrylic acid every day for 3 days after surgery.

Experimental Design

Wild-type male mice eyeballs were used in this study if their interocular refraction difference between the right and left eyes was <3.00 diopter and appeared to be uninjured and free of infection. The detailed flow chart of the experimental design is shown in [Supplemental Figure S2](#).

The time course of MMP-2 expression during myopia development was determined in 3-week-old wild-type mice. They were randomly divided into two groups: composed of mice that underwent monocular FD treatment for either 2 days, 1 week, or 2 weeks and age-matched mice with no FD (labeled as control).

To assess if the AAV8 could transfect the sclera effectively, sub-Tenon's capsule injection of AAV8-GFP was first performed in the 3-week-old mice. The eyes injected with AAV8-Vector served as the control. GFP was detected after 1 week of AAV8-GFP or AAV8-Vector injection.

To determine whether scleral MMP-2 up-regulation could induce myopia, 3-week-old wild-type mice ($n = 41$) were randomly divided into three groups: 14 were respectively injected with either AAV8-*Mmp2* or AAV8-Vector and another 13 noninjected mice served as the control (labeled as AAV8-*Mmp2*, AAV8-Vector, and control, respectively). Ocular measurements were recorded before AAV8 injection and 4 weeks later (week 3 and 7).

To determine the effect of scleral MMP-2 knockdown on the normal refraction development, 3-week-old wild-type mice ($n = 44$) were randomly assigned to three groups: 15 mice were injected with either shRNA-*Mmp2* or shRNA-scramble and another 14 noninjected mice served as the control (labeled as shRNA-*Mmp2*, shRNA-scramble, and control, respectively). Ocular measurements were recorded before AAV8 injection and 4 weeks later (week 3 and 7).

To determine the effect of scleral MMP-2 knockdown on FDM development, 3-week-old wild-type mice ($n = 60$) were randomly assigned to three groups: 23 mice were injected with either shRNA-*Mmp2* or shRNA-scramble, and after a week FDM was initiated for the next 4 weeks (labeled as shRNA-*Mmp2* + FD and shRNA-scramble + FD). Another 14 noninjected mice served as controls (labeled as Control + FD). Ocular measurements were measured before and 4 weeks after FDM (week 4 and 8).

To determine the effect of fibroblast-specific *Mmp2* deletion on the normal refractive development, ocular measurements were tracked from postnatal day (P)28 to P70 (4, 6, 8, and 10 weeks) in 12 *S100a4^{cre}Mmp2^{fl/fl}* and 25 *Mmp2^{fl/fl}* mice. To determine the effect of *S100a4^{cre}Mmp2^{fl/fl}* on FDM development, 16 *S100a4^{cre}Mmp2^{fl/fl}* and 18 *Mmp2^{fl/fl}* mice 4 weeks old were subjected to 2 weeks of FD (labeled as *S100a4^{cre}Mmp2^{fl/fl}* + FD and *Mmp2^{fl/fl}* + FD), and ocular measurements were performed before and 2 weeks after FD was initiated (4 and 6 weeks).

RNA Extraction, cDNA Preparation, and Quantitative Real-Time PCR

At the end of various treatment periods, the eyes were isolated. The sclera, cornea, and lens were dissected and homogenized separately in a ball mill, and total RNA was extracted using the RNeasy fibrous tissue minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total retinal RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After being treated with RQ1 RNase-Free DNase (Promega, Madison, WI), the RNAs of sclera, cornea, and retina were subjected to reverse transcription with random primers and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) to synthesize respective cDNAs.³⁷ RT-PCR was performed by using the specific primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) on an ABI 7500 Real-Time PCR system. The expression of each target mRNA relative to *18S rRNA* (sclera, retina, cornea, lens) was determined by using threshold cycle values and the $\Delta\Delta C_t$ method.³⁸ The identity of these above-mentioned primer sequences and primers used specifically for *S100a4^{cre}Mmp2^{fl/fl}* mice were confirmed with RT-PCR analysis and are provided in [Table 1](#).

Western Blot Analysis

Sclerae from different mice were homogenized in 150 μ L of ice-cold radiomunoprecipitation assay buffer (Beyotime, Shanghai, China) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (Beyotime) by using a ball mill, followed by sonication for approximately 30 to 60 seconds for complete tissue lysis. After being centrifuged at $13,000 \times g$ for 10 minutes at 4°C, the supernatant was aspirated and placed in a fresh tube on ice. The protein content was determined by using an Enhanced BCA Protein Assay Kit (Beyotime).

Equal amounts of proteins (50 μ g) from each sample were loaded onto 10% SDS-PAGE gel, separated by electrophoresis, and electrotransferred onto a nitrocellulose membrane (Millipore, Billerica, MA). After blocking with 5% nonfat milk for 2 hours at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. Primary antibodies against collagen type 1, $\alpha 1$ (Col1 $\alpha 1$; dilution 1:2000; ab88147; Abcam, Cambridge, MA), MMP-2 (dilution 1:1000; ab37150; Abcam), and α -tubulin (dilution 1:2000; ab52866; Abcam) were used. After washing thrice with tris-buffered saline with Tween-20 [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween-20] for 5 minutes each, a membrane was incubated with dilution 1:2000 IRDye 800CW goat anti-rabbit IgG (926-32211; Odyssey, Lincoln, NE) or dilution 1:2000 IRDye 800CW goat anti-mouse IgG (926-32210; Odyssey) antibodies for 2 hours at room temperature. The membrane was again washed in tris-buffered saline with Tween-20 thrice as before, followed by

Table 1 PCR Primers Used in This Study

Primer name	Forward primer	Reverse primer
Primers for RT-PCR		
18S	5'-CGGACACGGACAGGATTGAC-3'	5'-GTTCAAGCTGCCCGTCTCCTCATC-3'
MMP-2	5'-CAAGTTCCCCGGCGATGTC-3'	5'-TTCTGGTCAAGGTCACCTGTC-3'
MMP-2-CKO	5'-TGCTGAAAGATACCCTCAAGAAGAT-3'	5'-TATGTGATCTGGTTCTTGTCCAC-3'
Col1α1	5'-GAGAGCGAGGCCTTCCCGGA-3'	5'-GGGAGCCAGCGGGACCTTGT-3'
Primers for genotype identification of <i>MMP2^{fl/fl}</i> and <i>S100a4^{Cre}</i>		
5' <i>loxP</i>	5'-ATACCAGGCACATAGAGGAGAGGTC-3'	5'-GCTTCCTTGGGAATTGCCTTC-3'
3' <i>loxP</i>	5'-GCATCGCATTGTCTGAGTAGGTG-3'	5'-GCTGGTTGTAGTTTGGGGCAGA-3'
Null	5'-ATACCAGGCACATAGAGGAGAGGTC-3'	5'-GCTGGTTGTAGTTTGGGGCAGA-3'
<i>S100a4^{Cre}</i>	5'-GGATAGAGCACTTAAATGGGGA-3'	5'-GTGAAACAGCATTGCTGTCACTT-3'

densitometric analysis of the protein bands by using Image J version 1.48 software (NIH, Bethesda, MD). The values were normalized to corresponding loading control, α -tubulin. All Western blot analyses shown are representative of at least three independent experiments.

Immunofluorescence and Confocal Microscopy

Four-week-old *S100a4^{Cre}ROSA^{mT/mG}* and *ROSA^{mT/mG}* mice were sacrificed, and the eyes were isolated. The eyecups were then fixed in freshly prepared 4% paraformaldehyde for 20 minutes at room temperature and dehydrated in a gradient of 10% (2 hours), 20% (2 hours), and 30% (overnight) sucrose solutions at 4°C. The eyecups were embedded in Neg 50 Frozen Section Medium (Thermo Fisher Scientific, Waltham, MA), and the globes were then frozen in liquid nitrogen and stored at -80°C until sectioned. The eyecups were cut into 12- μ m-thick sections by using a freezing microtome. After rinsing the slides thrice in phosphate-buffered saline for 5 minutes each, they were then stained with DAPI (Vector Laboratories, Burlingame, CA). Neither tdTomato (red) nor GFP (green) required immunostaining for visualization. The frozen sections were visualized and photographed with a Zeiss LSM 710 confocal microscope (Zeiss).

To detect the GFP distribution in the sclera after 1 week of AAV8-GFP or AAV8-Vector injection, mice were sacrificed and the eyes were isolated. The eyecups were dissected to generate scleral flat-mount, then the specimens were fixed in freshly prepared 4% paraformaldehyde for 20 minutes at room temperature. After rinsing the slides thrice in phosphate-buffered saline for 5 minutes each, they were then stained with DAPI. The flat-mount sclera was visualized and photographed with a Zeiss LSM 710 confocal microscope.

Statistical Analysis

All data were analyzed by using statistical software (GraphPad Prism Software, version 7.0; GraphPad Inc., La Jolla, CA). A normality test (Shapiro-Wilk normality test)

analyzed the distribution of all data sets. For comparison of two groups, the differences were assessed by using parametric tests such as *t*-test (for normality of distributed data), nonparametric *U*-test, or Wilcoxon matched-pairs signed rank test. Multiple comparisons were performed by using one-way analysis of variance with Bonferroni's *post hoc* test (for normality of distributed data) or Kruskal-Wallis nonparametric with Dunn's *post hoc* test. Two-way analysis of variance for repeated measurements was used to compare the biometric measurements of the normal refractive development in the *S100a4^{Cre}Mmp2^{fl/fl}* and *Mmp2^{fl/fl}* mice, and the effect of genotype on time-dependent refractive development was evaluated with the Bonferroni *post hoc* test. Values of $P < 0.05$ were considered statistically significant.

Results

Scleral MMP-2 Expression Profile during Myopia Development

To evaluate the association between scleral MMP-2 expression and myopia, 3-week-old male wild-type C57BL/6 mice were subjected to FD for either 2 days, 1 week, or 2 weeks during which time scleral MMP-2 expression levels were measured. After 2 days of FD, the mRNA expression level of *Mmp2* in the sclera of FD-T eyes was significantly higher than in FD-F ($P < 0.05$) and control eyes ($P < 0.05$) (Figure 2A), whereas refraction was not statistically significant ($P = 0.66$) (Figure 2B). After 1 week of FD, a myopic shift in refraction occurred ($P < 0.001$) (Figure 2C). This myopic shift was accompanied by a further increase in *Mmp2* mRNA levels ($P < 0.01$) (Figure 2A) compared with the control eyes. After 2 weeks of FD, the mice became more myopic than that observed after 1 week ($P < 0.01$) (Figure 2D) with longer AL ($P < 0.05$) (Figure 2E) in FD-T eyes. Although the VCD of FD-T eyes increased slightly relative to FD-F eyes (Figure 2F), the difference was not statistically significant ($P = 0.07$). After 2 weeks of FD, the *Mmp2* mRNA level was significantly higher than in FD-F eyes ($P < 0.05$) and control eyes ($P < 0.05$) (Figure 2A).

Figure 2 Form deprivation (FD) induced rises in scleral matrix metalloproteinase (MMP)-2 expression. Right eye of each mouse in FD groups were subjected to FD (FD-T), and the left eyes served as the fellow controls (FD-F). Data from age-matched mice without FD induction are also presented for comparison (Control). **A:** Time course quantification from RT-PCR analysis of scleral *Mmp2* mRNA levels after 2 days, 1 week, and 2 weeks of FD; mRNA expression levels of *Mmp2* were normalized to those of Control. **B:** After 2 days of FD, no significant myopia was induced. **C:** After 1 week of FD, significant myopia was induced. **D–F:** After 2 weeks of FD, the FD-T eyes become increasingly myopic, accompanied by axial length (AL) elongation (**E**), a slight increase in vitreous chamber depth (VCD) was observed in the FD-T eyes (**F**), but there was no significant difference; the effects induced by FD are shown as interocular differences, that is, the difference between FD-T/right eyes and FD-F/left eyes (FD-T/right eyes – FD-F/left eyes). Data are expressed as means $\pm$ SEM (**A**). Box plot diagrams showing the distribution of the data. Line within the box indicates median; bars, range. $n = 8$ mice for each group at each time point (**A**); $n = 8$ control mice (**B**); $n = 12$ FD mice (**B**); $n = 10$ control mice (**C**); $n = 13$ FD mice (**C**); $n = 16$ control mice (**D–F**); $n = 15$ FD mice (**D–F**). * $P < 0.05$, ** $P < 0.01$. For the comparison of the *Mmp2* expression between FD-T and Control groups, unpaired *t*-test was used; for the comparison of the *Mmp2* expression difference between FD-T and FD-F groups, paired *t*-test was used (**A**). For the comparison of the interocular differences in refraction, AL, and VCD between the FD and Control groups, unpaired *t*-test or nonparametric *U*-test was used (**B–F**). D, diopter.

Like most other MMPs, MMP-2 was synthesized and secreted as an inactive latent form and activated by enzymatic cleavage in the extracellular environment. Accordingly, both the latent and active forms of MMP-2 in sclera were measured. In the FD-T eyes, the latent and active forms of MMP-2 levels in sclera were markedly higher than in the control and FD-F eyes after 2 days ($P < 0.05$) (Supplemental Figure S3, A and C) and 2 weeks ($P < 0.05$) (Supplemental Figure S3, B and D) of FD, which was consistent with the higher *Mmp2* mRNA expression that accompanied FD-induced scleral remodeling. After 2 weeks of FD, the scleral *Collα1* accumulation in FD-T eyes was significantly lower than in the control ($P < 0.05$) and FD-F ($P < 0.05$) eyes (Supplemental Figure S3, B and E).

Scleral MMP-2 Up-Regulation Induces Myopia

To determine the efficacy of AAV8-mediating gene transfer in sclera, the GFP in the flat-mount sclera was examined after 1 week of AAV8-GFP or AAV8-Vector injection. GFP was apparent in the whole sclera in the AAV8-GFP-injected eyes; however, no GFP was detected in the AAV8-Vector-injected eyes (Supplemental Figure S4).

To determine whether scleral MMP-2 up-regulation affected refractive development in wild-type mice, AAV8-*Mmp2* was injected into sub-Tenon's capsule of the right eyes. The scleral *Mmp2* gene expression level at 4 weeks after AAV8-*Mmp2* injection was significantly greater than that in the AAV8-Vector-injected eyes ($P < 0.001$) (Figure 3A). Similarly, both the latent ($P < 0.05$) and active ($P < 0.05$)

Figure 3 Selective promotion of *MMP2* gene expression by adeno-associated virus serotype 8 (AAV8)-*Mmp2* injection induces myopia. Right eye of each mouse (Right) was injected with AAV8-*Mmp2* or AAV8-Vector; the left eye (Left) served as the fellow control. Data from age-matched mice with no treatment are also presented for comparison (Control). **A–C:** RT-PCR analysis of *Mmp2* mRNA expression levels in sclera, retina, and cornea. **D:** No significant difference was observed in interocular differences in corneal radius curvature (CRC). **E:** RT-PCR analysis of *Col1α1* mRNA expression in sclera. Gene expression levels in parts **A–C** and **E** were normalized to those of Left of Control. **F:** AAV8-*Mmp2*–injected eyes become significantly myopic compared with the AAV8-Vector injected eyes. **G** and **H:** No significant differences in the interocular differences in axial length (AL) and vitreous chamber depth (VCD) were observed between the AAV8-Vector and AAV8-*Mmp2* groups. Data are expressed as means $\pm$ SEM (**A–C** and **E**). Box plot diagrams showing the distribution of the data. Line within the box indicates median; bars, range; **black dots**, outliers. $n = 7$ control mice (**A–C** and **E**); $n = 8$ AAV8-vector mice (**A–C** and **E**); $n = 8$ AAV8-*Mmp2* mice (**A–C** and **E**); $n = 13$ control mice (**D** and **F–H**); $n = 14$ AAV8-vector mice (**D** and **F–H**); $n = 14$ AAV8-*Mmp2* mice (**D** and **F–H**). ** $P < 0.01$, *** $P < 0.001$. Differences among Control, AAV8-Vector, and AAV8-*Mmp2* groups were assessed by using one-way analysis of variance with Bonferroni's *post hoc* test (for normality distributed data) or Kruskal-Wallis nonparametric with Dunn's *post hoc* test. D, diopter.

forms of *MMP-2* in sclera were markedly greater in the AAV8-*Mmp2*–injected eyes than in the AAV8-Vector–injected eyes (Supplemental Figure S5, A and B). No significant difference in retinal *Mmp2* mRNA expression was observed between the AAV8-*Mmp2*– and AAV8-Vector–injected eyes ($P = 0.63$) (Figure 3B). Although a greater corneal *Mmp2* mRNA expression level was observed in AAV8-*Mmp2*–injected eyes than in the AAV8-Vector–injected eyes ($P < 0.01$) (Figure 3C), no significant difference in the CRC was observed ($P = 0.95$) (Figure 3D), which suggested that elevation of corneal *MMP-2* expression did not have a significant effect on refraction.

The effect of *MMP-2* up-regulation on scleral *Col1α1* expression was evaluated because type I collagen accounts for approximately 90% of the scleral total dry weight. No significant difference in scleral *Col1α1* mRNA expression was observed between AAV8-*Mmp2*– and AAV8-Vector–injected eyes ($P = 0.80$) (Figure 3E). However, its protein expression was 28% lower in AAV8-*Mmp2*–injected eyes than in their noninjected fellow eyes ($P < 0.01$) (Supplemental Figure S5, A and C), whereas AAV-Vector injection had no significant effect on *Col1α1* protein expression ($P = 0.31$) (Supplemental Figure S5, A and C).

Baseline measurements of all biometric variables were similar among the three groups and between eyes of individual mice in the same group. No significant differences in any of these variables were detected between the control and fellow eyes of all groups at any time. Thus, the interocular differences in refraction and axial components between injected eyes and their fellow eyes (injected/right eyes – their fellow/left eyes) were used to assess the biometric changes after AAV8 injection. Data from control groups are also shown for comparison.

Scleral *MMP-2* up-regulation resulted in a myopic shift in refraction in the AAV8-*Mmp2*–injected eyes 4 weeks after injection compared with AAV8-Vector–injected eyes ($P < 0.001$) (Figure 3F). However, AL ($P = 0.49$) and VCD ($P = 0.70$) differences were not statistically significant (Figure 3, G and H). This disconnect between changes in refraction and ocular biometric invariance may be due to the limited resolving power of optical coherence tomograph to identify any biometric changes if they fell below the level of instrument detection.³⁹

Taken together, scleral *MMP-2* up-regulation induced a significant myopic shift in refraction but left AL and VCD unaltered.

Scleral MMP-2 Knockdown Suppresses FDM Development without Altering Normal Refractive Development

Four weeks after shRNA-*Mmp2* injection, the scleral *Mmp2* gene expression level was significantly lower than in shRNA-scramble-injected eyes ($P < 0.001$), (Figure 4A). However, no significant difference was observed in retinal *Mmp2* mRNA expression between the shRNA-*Mmp2*- and shRNA-scramble-injected eyes ($P = 0.90$) (Figure 4B). Although shRNA-*Mmp2* injection lowered *Mmp2* mRNA expression compared with shRNA-scramble-injected eyes ($P < 0.01$) (Figure 4C), no significant difference in CRC was observed ($P = 0.96$) (Figure 4D). No significant differences were observed in interocular differences in refraction ($P = 0.20$), AL ($P = 0.29$), and VCD ($P = 0.91$) between the shRNA-scramble and shRNA-*Mmp2* groups (Figure 4, E–G). Similarly, no significant difference was observed in scleral *Col1a1* mRNA expression between these two groups ($P = 0.67$) (Figure 4H). Therefore, reducing the scleral *Mmp2* expression level had no effect on either *Col1a1* gene expression level or normal refraction development in a normal visual environment.

To determine whether reduced scleral MMP-2 expression inhibited myopia development, FD was initiated 1 week after injecting either shRNA-*Mmp2* or shRNA-scramble. After 4 weeks of FD, in the Control + FD group *Mmp2* mRNA expression in FD-T eyes was significantly higher than in the FD-F eyes ($P < 0.01$), whereas *Mmp2* mRNA expression in the FD-T eyes of the shRNA-*Mmp2* + FD group was significantly lower than in the FD-T eyes of the shRNA-scramble + FD group ($P < 0.001$) (Figure 5A). These effects on gene expression levels paralleled higher MMP-2 active form levels in the FD-T eyes than in the FD-F eyes of the Control + FD group ($P < 0.05$), whereas this increase was suppressed in the FD-T eyes of the shRNA-*Mmp2* + FD group compared with the FD-T eyes of the shRNA-scramble + FD group ($P < 0.05$) (Supplemental Figure S6, A and B). After 4 weeks of FD, significant myopia was induced in all three groups, but inhibition of scleral MMP-2 expression by shRNA-*Mmp2* inhibited the myopic refraction by approximately 23% compared with the shRNA-scramble + FD group ($P < 0.05$) (Figure 5B). In parallel with the changes in refraction, AL elongations ($P < 0.05$) and increases in VCD ($P < 0.05$) were also significantly lower in the shRNA-*Mmp2* + FD group than in

Figure 4 Effects of sub-Tenon's capsule injection of shRNA-*Mmp2* on matrix metalloproteinase (MMP)-2 expression and ocular variables 4 weeks after injection. Right eye of each mouse (Right) was injected with shRNA-*Mmp2* or shRNA-scramble; the left eye (Left) served as the fellow control. Data from age-matched mice with no treatment are also presented for comparison (Control). **A–C:** RT-PCR analysis of *Mmp2* mRNA expression in sclera, retina, and cornea. **D–G:** No significant differences were observed in interocular differences in corneal radius curvature (CRC), refraction, axial length (AL), and vitreous chamber depth (VCD) between the shRNA-*Mmp2* and shRNA-scramble groups. **H:** RT-PCR analysis of *Col1a1* mRNA expression in sclera. Data are expressed as means $\pm$ SEM (**A–C** and **H**). Gene expression levels in panels **A–C** and **H** were normalized to those of Left of Control. Line within the box indicates median; bars, range; **black dots**, outliers. $n = 6$ control mice (**A–C** and **H**); $n = 7$ shRNA-scramble mice (**A–C** and **H**); $n = 7$ shRNA-*Mmp2* mice (**A–C** and **H**); $n = 14$ control mice (**D–G**); $n = 15$ shRNA-scramble mice (**D–G**); $n = 15$ shRNA-*Mmp2* mice (**D–G**). $^{**}P < 0.01$, $^{***}P < 0.001$. Differences among Control, shRNA-scramble, and shRNA-*Mmp2* groups were assessed by using one-way analysis of variance with Bonferroni's *post hoc* test (for normality distributed data) or Kruskal-Wallis nonparametric with Dunn's *post hoc* test. D, diopter.

Figure 5 Scleral matrix metalloproteinase (MMP)-2 down-regulation inhibits form deprivation myopia (FDM) development. Right eye of each mouse was injected with shRNA-*Mmp2* or shRNA-scramble, and 1 week after injection FD was imposed for a subsequent 4 weeks (FD-T) and the left eyes (FD-F) served as the fellow controls; the form-deprived noninjected mice (Control + FD) served as the control. **A**: RT-PCR analysis of the scleral *Mmp2* mRNA expression. **B–D**: The myopia induced in the shRNA-*Mmp2* + FD group was less than in the shRNA-scramble + FD group, which was accompanied by less axial length (AL) elongation and smaller increases in vitreous chamber depth (VCD). **E**: RT-PCR analysis of the scleral *Col1a1* mRNA expression. **F**: No significant difference was observed in corneal radius curvature (CRC). Data are expressed as means $\pm$ SEM (**A** and **E**). Gene expression levels in parts **A** and **E** were normalized to those of Left of Control + FD. Line within the box indicates median; bars, range. $n = 9$ control mice (**A** and **E**); $n = 15$ AAV8-Vector mice (**A** and **E**); $n = 15$ AAV8-*Mmp-2* mice (**A** and **E**); $n = 14$ control mice (**B–D** and **F**); $n = 23$ AAV8-vector mice (**B–D** and **F**); $n = 23$ AAV8-*Mmp-2* mice (**B–D** and **F**). * $P < 0.05$, one-way analysis of variance with Bonferroni's *post hoc* test; ** $P < 0.01$, for Control + FD group: FD-F versus FD-T, paired *t*-test; *** $P < 0.001$. Differences among Control + FD, shRNA-scramble, and shRNA-*Mmp2* groups were assessed by using one-way analysis of variance with Bonferroni's *post hoc* test. Differences in the scleral *Col1a1* mRNA expression were assessed by using Kruskal-Wallis nonparametric with Dunn's *post hoc* test. D, diopter.

the shRNA-scramble + FD group (Figure 5, C and D). No significant difference in scleral *Col1a1* mRNA expression was observed between the shRNA-*Mmp2* and shRNA-scramble groups ($P = 0.73$) (Figure 5E). Nevertheless, *Col1a1* protein expression in FD-T eyes was 25% lower than in the FD-F eyes of shRNA-scramble injected mice ($P < 0.01$), whereas this decline was not observed in the

FD-T eyes of the shRNA-*Mmp2* + FD group ($P = 0.09$) (Supplemental Figure S6, A and C). In addition, no significant difference in the CRC was observed between the shRNA-*Mmp2* + FD and shRNA-scramble + FD groups ($P = 0.39$) (Figure 5F). Therefore, reducing scleral MMP-2 expression suppressed both FDM development and the decline in *Col1a1* accumulation.

Taken together, reducing scleral MMP-2 expression had no effect on normal refractive development but attenuated declines in *Col1a1* accumulation and FDM development.

Fibroblast-Specific *Mmp2* Deletion Lessens FDM

Scleral fibroblasts are the predominant cell population in mammalian sclera and they are a primary source of MMP-2 expression. To confirm that reducing scleral MMP-2 contributed to a decline in myopia development, the *Cre-loxP* system was used to generate a novel fibroblast-specific *Mmp2* deletion mouse model (Figure 1A).

To determine the distribution of *S100a4* promoter driving Cre recombinase expression in the sclera, the GFP location in flat-mount sclera in *S100a4^{cre}ROSA^{mT/mG}* and *ROSA^{mT/mG}* mice was assessed. Strong tdTomato fluorescence (red) was observed in all of the tissue (including the retina, cornea, sclera, and lens) in the *ROSA^{mT/mG}* (Supplemental Figure S7A) and *S100a4^{cre}ROSA^{mT/mG}* (Supplemental Figure S7B) mice. The GFP expression identified the location of Cre recombinase expression. It was distributed in the whole sclera of the *S100a4^{cre}ROSA^{mT/mG}* mice (Supplemental Figure S7B) and also in the cornea and lens of the *S100a4^{cre}ROSA^{mT/mG}* mice (Supplemental Figure S7B).

Scleral MMP-2 knockdown efficiency obtained by fibroblast-specific *Mmp2* deletion was significantly lower in the *S100a4^{cre}Mmp2^{fl/fl}* mice than in the *Mmp2^{fl/fl}* mice ($P < 0.05$) (Figure 6A). The selectivity of this procedure in knocking down scleral *Mmp2* gene expression was apparent

from the lack of a difference between *Mmp2* expression levels in the retina ($P = 0.68$) and cornea ($P = 0.20$) in the *S100a4^{cre}Mmp2^{fl/fl}* and *Mmp2^{fl/fl}* mice (Figure 6A); however, the lens *Mmp2* expression level in *S100a4^{cre}Mmp2^{fl/fl}* mice was significantly lower than in *Mmp2^{fl/fl}* mice ($P < 0.01$) (Figure 6A). No significant difference was found in scleral *Col1a1* mRNA expression between these two different genotypes ($P = 0.23$) (Figure 6B). Even though hyperopia developed in both genotypes from P28 to P70 (4 to 10 weeks) in a normal visual environment, the *S100a4^{cre}Mmp2^{fl/fl}* mice were less hyperopic than the *Mmp2^{fl/fl}* mice ($P < 0.001$) (Figure 6C). Significant differences in refraction developed at P28 ($P < 0.01$), P42 ($P < 0.05$), P56 ($P < 0.05$), and P70 ($P < 0.05$) between the two genotypes (Figure 6C). The AL increased more in the *S100a4^{cre}Mmp2^{fl/fl}* mice than in the *Mmp2^{fl/fl}* mice (Figure 6D). Significant differences in AL developed at P28 ($P < 0.05$), P42 ($P < 0.01$), P56 ($P < 0.01$), and P70 ($P < 0.01$) between the two genotypes (Figure 6D). The LT increased more in the *S100a4^{cre}Mmp2^{fl/fl}* mice than in the *Mmp2^{fl/fl}* mice (Figure 6E). Significant differences in AL developed at P28 ($P < 0.05$), P42 ($P < 0.01$), P56 ($P < 0.05$), and P70 ($P < 0.05$) between the two genotypes (Figure 6E).

To determine whether fibroblast-specific *Mmp2* deletion could suppress myopia development, 4-week-old *S100a4^{cre}Mmp2^{fl/fl}* and *Mmp2^{fl/fl}* mice were subjected to FD for 2 weeks. Because the baseline values in the refraction, AL, and LT components of these two genotypes were different (Figure 6, C–E), comparing raw data of these two

Figure 6 Retardation of refractive development in *S100a4^{cre}Mmp2^{fl/fl}* mice. **A:** RT-PCR analysis of *Mmp2* mRNA expression levels in sclera, cornea, retina, and lens in *S100a4^{cre}Mmp2^{fl/fl}* and *Mmp2^{fl/fl}* mice. **B:** RT-PCR analysis of *Col1a1* mRNA expression levels in sclera. Gene expression levels in **A** and **B** were normalized to those of *Mmp2^{fl/fl}*. **C–E:** A significant myopic shift in refraction (**C**), greater axial length (AL; **D**), and greater lens thickness (LT; **E**) were observed in *S100a4^{cre}Mmp2^{fl/fl}* mice than in *Mmp2^{fl/fl}* mice at the indicated postnatal time points during normal refractive development. Data are expressed as means $\pm$ SEM (**A**). Line within the box indicates median; bars, range. $n = 12$ *S100a4^{cre}Mmp2^{fl/fl}* mice (**C–E**); $n = 25$ *Mmp2^{fl/fl}* mice (**C–E**); $n = 12$ sclera (**A**); $n = 5$ cornea (**A**); $n = 5$ retina (**A**); $n = 12$ lens (**A**); $n = 12$ *Mmp2^{fl/fl}* mice (**B**); $n = 12$ *S100a4^{cre}Mmp2^{fl/fl}* mice (**B**). * $P < 0.05$; ** $P < 0.01$, determined by *U*-test or unpaired *t*-test (**A** and **B**); *** $P < 0.001$, determined by two-way analysis of variance for repeated measurements for comparing the refraction and AL in *S100a4^{cre}Mmp2^{fl/fl}* and the *Mmp2^{fl/fl}* mice, the effect of genotype on time-dependent refractive development was evaluated by the Bonferroni *post hoc* test or Kruskal–Wallis nonparametric with Dunn's *post hoc* test (**C–E**). D, diopter.

Figure 7 Fibroblast-specific *Mmp2* deletion attenuates form deprivation myopia (FDM) (*S100a4*^{cre}*Mmp2*^{fl/fl}) mice. **A** and **B**: RT-PCR analysis of scleral *Mmp2* and *Col1a1* mRNA expression of form-deprived eyes (FD-T) and fellow eyes (FD-F) in *S100a4*^{cre}*Mmp2*^{fl/fl} + FD and *Mmp2*^{fl/fl} + FD mice. Gene expression levels in **panels A** and **B** were normalized to those of FD-F of *Mmp2*^{fl/fl} + FD mice. **C**: The myopia induced in the *S100a4*^{cre}*Mmp2*^{fl/fl} + FD group was approximately 27% lower than in the *Mmp2*^{fl/fl} + FD group. **D–F**: Interocular differences in axial length (AL; **D**), vitreous chamber depth (VCD; **E**), and lens thickness (LT; **F**) between *S100a4*^{cre}*Mmp2*^{fl/fl} + FD and *Mmp2*^{fl/fl} + FD groups were not statistically different. Data are expressed as means $\pm$ SEM (**A** and **B**). Line within the box indicates median; bars, range; **black dots**, outliers. $n = 6$ *Mmp2*^{fl/fl} + FD mice (**A** and **B**); $n = 6$ *S100a4*^{cre}*Mmp2*^{fl/fl} + FD mice (**A** and **B**); $n = 16$ *Mmp2*^{fl/fl} + FD mice (**C–F**); $n = 19$ *S100a4*^{cre}*Mmp2*^{fl/fl} + FD mice (**C–F**). * $P < 0.05$, determined by nonparametric *U*-test; ** $P < 0.01$, for *Mmp2*^{fl/fl} + FD group between FD-F versus FD-T, paired *t*-test or Wilcoxon matched-pairs signed rank test; for FD-T eyes between *S100a4*^{cre}*Mmp2*^{fl/fl} + FD and *Mmp2*^{fl/fl} + FD groups, unpaired *t*-test or *U*-test. D, diopter.

groups may be misleading. Thus, the interocular differences (FD-T – FD-F) in each mouse were instead used in the analysis.

After 2 weeks of FD, the mRNA expression ($P < 0.01$) (Figure 7A) and the latent ($P < 0.05$) and active ($P < 0.05$) forms of MMP-2 levels (Supplemental Figure S8, A and B) in sclera in FD-T eyes were significantly higher than in *Mmp2*^{fl/fl} mice but not in *S100a4*^{cre}*Mmp2*^{fl/fl} mice in comparison with respective levels in their FD-F eyes. The mRNA ($P < 0.05$) and active form ($P < 0.05$) of MMP-2 in FD-T eyes were significantly lower in *S100a4*^{cre}*Mmp2*^{fl/fl} mice than in *Mmp2*^{fl/fl} mice (Figure 7A, Supplemental Figure S1, A and B). In addition, no significant difference was observed in *Colla1* mRNA expression level of FD-T eyes between the two groups ($P = 0.36$) (Figure 7B), whereas its protein expression in FD-T eyes was significantly higher in the *S100a4*^{cre}*Mmp2*^{fl/fl} mice than in the *Mmp2*^{fl/fl} mice ($P < 0.05$) (Supplemental Figure S8, A and C). Myopia induced in the *S100a4*^{cre}*Mmp2*^{fl/fl} mice was 27% less than in the *Mmp2*^{fl/fl} mice ($P < 0.05$) (Figure 7C). Both the AL elongation ($P = 0.17$) (Figure 7D) and increase in VCD ($P = 0.05$) (Figure 7E) were inhibited in *S100a4*^{cre}*Mmp2*^{fl/fl} mice slightly more than in the *Mmp2*^{fl/fl} mice, but these differences were not significant. No significant differences in the interocular difference in LT was observed between *S100a4*^{cre}*Mmp2*^{fl/fl} mice and *Mmp2*^{fl/fl} mice ($P = 0.65$) (Figure 7F).

Taken together, reducing the scleral MMP-2 expression level by fibroblast-specific *Mmp2* deletion suppressed both FDM and the declines in *Col1a1* accumulation relative to those in the *Mmp2*^{fl/fl} mice.

Discussion

This study demonstrates that there is a cause-and-effect relationship between MMP-2 up-regulation and myopia induced by FD in an experimental mouse model. Myopia initiation depended on increases in MMP-2 expression because MMP-2 expression levels were the same in the fellow and noninjected eyes in a normal visual environment, whereas FD induced up-regulation. Furthermore, the FD-induced increases in MMP-2 gene and protein expression levels preceded any detectable changes in refraction, AL, and VCD. Another finding consistent with this notion is that MMP-2 up-regulation resulting from AAV8 gene transfer induced myopia, whereas declines in MMP-2 expression induced by shRNA suppressed FDM. This inhibitory effect on FDM was confirmed by showing that in *S100a4*^{cre}*Mmp2*^{fl/fl} mice this response was also suppressed to the same extent as in the MMP2 shRNA-injected mice. These results agree with a prior study showing that MMP-2 is a viable target to inhibit myopia development.⁴⁰ Despite all of the evidence supportive of a cause-and-effect relationship between increases in

MMP-2 expression levels and myopia, it is still possible that there is an unknown confounder inducing in parallel these responses to form deprivation.

However, measuring the biometric variables accompanying changes caused by FD is a daunting task in mice because of their small eyes. This limitation makes it difficult to resolve differences, whereas it was possible to identify significant changes in the larger eyes of other experimental models.⁴¹ Our inability to detect significant changes in AL and VCD shown in Figures 2D, 3, G and H, and 5, E and F, is possibly attributable to the inability of optical coherence tomograph methods to resolve small changes in AL and VCD when the refraction change is small in mice.

MMP-2 involvement in mediating myopia development is based on previous studies showing that its up-regulation is associated with declines in scleral biomechanical strength and augmentation of this condition.¹⁰ Because selective MMP-2 inhibitors were unavailable, it was previously not possible to determine whether there was a cause-and-effect relationship between MMP-2 up-regulation and initiation as well as promotion of myopia development. However, we were able to selectively increase and decrease MMP-2 expression by manipulating its gene and protein expression levels. Specifically, MMP-2 up-regulation reduced type I collagen accumulation and induced myopia development, whereas declines in MMP-2 expression obtained by injecting *Mmp2* gene shRNA function hindered myopia development. Similarly, in a conditional fibroblast *Mmp2* deletion system a decline in scleral MMP-2 expression had effects similar to those obtained with the aforementioned shRNA system. Therefore, MMP-2-induced increases in collagen degradation underlying myopia appear to precede declines in scleral biomechanical strength identified in human sclera and other experimental animal models of this condition.

Even though genetic manipulations in mice identified several genes, including *Egr1*, *Irbp*, *A2AR*, and *Rar*^{42–45} whose expression modulation affects refractive development by using body-wide gene knockout mice, the identity of the cell type in which such a change occurred could not be determined. To deal with this limitation, the present study used a fibroblast-specific *Mmp2* knockout model to determine the contribution made by scleral MMP-2 up-regulation to myopia development. Using such a fibroblast-specific knockout could possibly clarify if MMP-2 release by other cell types besides fibroblasts affects myopia progression.⁴⁶ However, it was not possible to resolve the contribution made by fibroblast MMP-2 expression to modulating myopia because the knockdown efficiency of *Mmp2* expression in the fibroblast conditional knockout was relatively small. Another hindrance is that it is uncertain whether expression of MMP-2 by other cell types masked the effects of gain and loss of fibroblast-derived MMP-2 function on FDM development.

To the best of our knowledge, this is the first time a fibroblast-specific gene knockout mouse was developed with the intent to determine the contributory role of a single

gene on refractive development and FDM progression. Even though our results indicate that increases in MMP-2 expression contribute to rises in type I collagen degradation and promotion of myopia development, in some cases the changes in MMP-2 expression and myopia progression do not correspond with one another. This occurred after shRNA-*Mmp2* injection in which the active form of MMP-2 declined by 54%, whereas myopia development declined by just 23% (Supplemental Figure S6B and Figure 5B). Nevertheless, there is no reason to expect that there would be a one-to-one correspondence between changes in mRNA expression levels and FDM. Another consideration is that the lack of correspondence could be due to the involvement of other proteases besides MMP-2 inducing changes in myopia development.¹⁹ In the *S100a4^{cre}Mmp2^{fl/fl}* mice, the active form of MMP-2 in sclera declined by 20%, whereas myopia development declined by 27% (Supplemental Figure S8B and Figure 7C). However, this correspondence could be fortuitous because it is possible that there are other cell types besides fibroblasts in which *Mmp2* gene expression is left unaltered by fibroblast-specific *Mmp2* deletion. In addition, there could be compensatory effects resulting from up-regulation of other proteases induced by FD contributing to scleral remodeling. Another question pertains to the limited MMP-2 knockdown efficiency obtained in *S100a4^{cre}Mmp2^{fl/fl}* mice. One possibility for such a low efficiency could be due to limited fibroblast expression of the *S100a4* promoter driving Cre recombinase expression.

Conclusions

This study used gain and loss of MMP-2 function strategies to confirm that changes in scleral MMP-2 expression directly contribute to modulating myopia in mice. The results show that increases in MMP-2 expression precede increases in FDM that stem from increases in both type I collagen degradation and presumably ECM remodeling. Because MMP-2 up-regulation had effects that were opposite to those caused by inhibiting MMP-2 expression, this result confirms that a cause-and-effect relationship exists between changes in MMP-2 expression and myopia in mice. These findings point to the possibility that developing selective MMP-2 inhibitors could be a viable approach in the future to improve management of myopia in a clinical setting.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.04.011>.

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