The Effect of TIMP-1 on the Cone Mosaic in the Retina of the Rat Model of Retinitis Pigmentosa

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PURPOSE. The array of photoreceptors found in normal retinas provides uniform and regular sampling of the visual space. In contrast, cones in retinas of the S334ter-line-3 rat model for RP migrate to form a mosaic of rings, leaving large holes with few or no photoreceptors. Similar mosaics appear in human patients with other forms of retinal dystrophy. In the current study, we aimed to investigate the effect of tissue inhibitor of metalloproteinase-1 (TIMP-1) on the mosaic of cones in S334ter-line-3 rat retinas. We focused on TIMP-1 because it is one of the regulators of the extracellular matrix important for cellular migration.

METHODS. Immunohistochemistry was performed to reveal M-opsin cone cells (M-cone) and the results were quantified to test statistically whether or not TIMP-1 restores the mosaics to normal. In particular, the tests focused on the Voronoi and nearest-neighbor distance analyses.

RESULTS. Our tests indicated that TIMP-1 led to significant disruption of the M-opsin cone rings in S334ter-line-3 rat retinas and resulted in almost complete homogeneous mosaics. In addition, TIMP-1 induced the M-cone spatial distribution to become closer to random with decreased regularity in S334ter-line-3 rat retinas.

CONCLUSIONS. These findings confirm that TIMP-1 induced M-cone mosaics in S334ter-line-3 to gain homogeneity without reaching the degree of regularity seen in normal retinal mosaics. Even if TIMP-1 fails to promote regularity, the effects of this drug on homogeneity appear to be so dramatic that TIMP-1 may be a potential therapeutic agent. TIMP-1 improves sampling of the visual field simply by causing homogeneity.

Keywords: cones, retinitis pigmentosa, TIMP-1, mosaic

The outer nuclear layer (ONL) of the vertebrate retina contains a tightly packed, uniform array of rods and cones, which is essential to ensure that the visual world is regularly sampled with no empty visual space. The density of rods constrains visual sensitivity and the spacing of cones determines resolution and thus acuity of vision.¹ Past studies have described that regular and homogeneous spacing of photoreceptors, as seen in some mammalian species and zebrafish,²⁻⁸ are important for sampling the visual space efficiently.^{9,10} However, cones in the S334ter-line-3 rat model of RP were recently shown both to survive for a longer period of time after the early rod deaths and to remodel in their mosaic pattern into orderly arrays of rings.¹¹⁻¹³ Similar dark patches (i.e., holes) are noted in several human eye diseases caused by retinal dystrophy, inherited retinal degeneration, and photo-pigment genetic perturbations in M-cones.¹⁴⁻¹⁷ The centers of these rings lack photoreceptors, indicating local loss of visual function. Consequently, knowledge on modulating and rear-

ranging photoreceptors from the ring patterns into more regular and homogeneous distribution would help improve conditions in these patients.

In past studies, it has been reported that the balance in the level of enzymes that mediate the degradation of the extracellular matrix (ECM) is important for modulation of migration of neurons, including photoreceptors.¹⁸⁻²⁰ In mammals, these enzymes are the metalloproteinase (MMP; degrades ECM)²¹ and its natural inhibitor, tissue inhibitor of metalloproteinase (TIMP),²² and together, they modulate neural organization by remodeling and organizing of ECM in normal and pathological retinas.^{23,24} In particular, a previous study showed that TIMP-1 applied to co-cultured rat retinal neurons with human retinal epithelial cells led to modulation of photoreceptor migration.¹⁹ Also, opposite from some other members of the TIMP families, TIMP-1 does not inhibit endothelial cell migration. Among members of the MMP and TIMP families, MMP-9 and its inhibitor, TIMP-1, are predomi-

nantly expressed in the interphotoreceptor matrix (IPM).²⁵ This indicates that TIMP-1 may play a role in modulating turnover of IPM, which is important for various photoreceptor functions and maintenance.^{26–33}

In human and animal models with various ocular diseases, including retinal degeneration, the level of TIMP-1 is significantly upregulated.^{34–36} Positive correlation between TIMP-1 expression and tumor growth in several cell lines indicate that TIMP-1 also may play a key role as a survival factor.^{37–41} It was proposed that TIMP-1 may protect ECM-bound growth factors critical for cell survival.²⁴

In the present study, we investigated if exogenous application of the TIMP-1 could affect the mosaic of cones in S334ter-line-3 rat retinas. Because we studied the effects of TIMP-1 on the mosaic of cones, we needed statistical tools to compare the spatial distribution of these cells in different conditions.⁴² One of the most commonly used statistical measures is the areas of Voronoi domains: regions of space obtainable by enclosing each cell in the mosaic in space closest to itself than any other cells. Another statistical analysis focused on the nearest-neighbor distance (NND), the distance to the closest neuron for every cell.⁴³ Using these analyses, we report for the first time that the use of TIMP-1 brings statistically significant changes to the cone mosaic in S334ter-line-3, allowing it to become similar to that in normal retinas in their homogeneity. Ultimately, deeper understanding of the action of TIMP-1 could help future therapeutics against various eye diseases, where cone mosaic remodeling would benefit.

MATERIALS AND METHODS

Animals

The third line of albino Sprague-Dawley rats homozygous for the truncated murine opsin gene (created a stop codon at Serine residue 334; S334ter-line-3) was obtained from Matthew LaVail, PhD (University of California, San Francisco, CA, USA). Homozygous S334ter-3 male rats are mated with homozygous S334ter-3 female rats to produce offspring for the S334ter-3 transgene that are used throughout this study. For control, age-matched Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were used. All rats were housed under cyclic 12/12-hour light/dark conditions with free access to food and water. Both sexes of normal (control) and S334ter-line-3 rats were used. This model shall be referred to as the RP model in the rest of the article. Animals were treated in accordance with the regulations of the Veterinary Authority of University of Southern California and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Administration of TIMP-1

Tissue inhibitor of metalloproteinase-1 (Sigma-Aldrich Corp., St. Louis, MO, USA) was prepared in sterile-filtered PBS, adjusted to pH 7.4, and sterile-filtered before administration. Tissue inhibitor of metalloproteinase-1 was administered by intravitreal injection with a fine glass microelectrode through the sclera at the level of the temporal peripheral retina. For preliminary testing, 4 μ L of several different final concentrations of the TIMP-1 (10, 25, and 50 μ g/mL) were applied on normal and RP rats at postnatal day (P)20, P30, P45, and P60. Survival periods of 1 to 3 hours, 3 and 5 days, and 1 to 6 weeks were tested. Both 25 and 50 μ g/mL gave similar end results in terms of the degree of change in the mosaics of M-opsin-immunostained reactive cones (termed M-cones), thus 4 μ L 25 μ g/mL was used for the rest of the experiments. It was also determined that the optimal stage for the injection of TIMP-1

was P45, the age when cones are arranged in rings across the entire retina.¹² As for survival periods, 1 hour, 2 weeks, and 6 weeks were used, as they best described the progress of cone mosaic changes with application of TIMP-1. Sham injections, for controls, consisted of 4 μ L of the same sterile-filtered PBS used to prepare the TIMP-1. For each animal, one eye was used to inject TIMP-1 while the other was used to inject saline for comparison. Surgeries on rats were performed under anesthesia induced by intraperitoneal injection of ketamine (100 mg/kg; KETASET, Fort Dodge, IA, USA) and xylazine (20 mg/kg, X-Ject SA; Butler, Dublin, OH, USA). The entire injection procedure required only a few minutes, allowing us to finish before the animals recovered from anesthesia.

Tissue Preparation

Animals at P45 (1-hour survival period), P59 (2-week survival period), and P87 (6-week survival period) were used ($n = 15$ for each stage). Animals were deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg body weight) and the eyes were enucleated. Animals were then killed with an overdose of pentobarbital. The anterior segment and crystalline lens were removed and the eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 30 minutes to 1 hour at 4°C. Following fixation, the retinas were carefully isolated from the eyecups and were transferred to 30% sucrose in PB for 24 hours at 4°C. For storage, all retinas were then frozen in liquid nitrogen, and stored at –70°C, thawed, and rinsed in 0.01 M PBS (pH 7.4). For cryostat sections, eyecups were embedded in optimal cutting temperature embedding medium (Tissue-Tek, Elkhart, IN, USA), then quickly frozen in liquid nitrogen and subsequently sectioned along the vertical meridian on a cryostat at a thickness of 20 μ m.

Immunohistochemistry

For immunohistochemistry, 20- μ m-thick cryostat sections were incubated in 10% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, Inc.) for 1 hour at room temperature. Sections were then incubated overnight with rabbit polyclonal antibody directed against glial fibrillary acidic protein (GFAP) (Sigma-Aldrich Corp.). This antiserum was diluted in PBS containing 0.5% Triton X-100 at 4°C. Retinas were washed in PBS for 45 minutes (3 $\times$ 15 minutes) and afterward incubated for 2 hours at room temperature in carboxymethylindocyanine-3 (Cy3)-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Next, the sections were washed for 30 minutes with 0.1M PB and coverslipped with Vectashield mounting medium (Vector Labs, Burlingame, CA, USA). For whole-mount immunostaining, the same immunohistochemical procedures described above were used. However, incubation times with the primary antibodies were longer (2 nights with rabbit polyclonal antibody directed against middle-wavelength-sensitive opsin [M-opsin],¹³ mouse monoclonal antibody directed against glutamine synthetase [GS; Chemicon, Temecula, CA, USA]) and so were those with the secondary antibodies (1 night either with Cy3-conjugated donkey anti-rabbit IgG or with Alexa 488 donkey anti-mouse IgG).

For double-label studies, whole mounts were incubated for 2 nights in a mixture of anti-M-opsin and anti-GS markers. Incubation with these antibodies used 0.5% Triton X-100 in 0.1 M PBS at 4°C. After this incubation, whole mounts were rinsed for 30 minutes with 0.1 M PBS. Afterward, we incubated them with Cy3-conjugated donkey anti-rabbit IgG and Alexa 488 donkey anti-mouse overnight at 4°C. Whole mounts were then

washed again for 30 minutes with 0.1 M PB and coverslipped with Vectashield mounting medium. Sections and whole mounts were then analyzed using a Zeiss LSM 510 (Zeiss, NY, USA) confocal microscope. Immunofluorescence images were processed with the Zeiss LSM-PC software. Finally, the brightness and contrast of the images were adjusted using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA). All Photoshop adjustments were carried out equally across sections.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

Cell death was visualized by a modified TUNEL technique, according to the manufacturer's instructions (In Situ Cell Detection kit; Boehringer Mannheim, Mannheim, Germany). The P45, P59, and P87 cryostat sections of normal retinas from the control and TIMP-1 groups were incubated with Proteinase K (10 µg/mL in 10 mM Tris/HCl, pH 7.4–8.0) for 10 minutes at 37°C. The sections were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase plus nucleotide mixture in reaction buffer) for 60 minutes at 37°C. The sections were then washed again for 30 minutes with 0.1 M PB and coverslipped with Vectashield mounting medium.

Construction of Nuclei-Positions Map

Confocal micrographs of the retinas ($n = 3$ –5 animals for each group) were taken at the focal level of the nuclei of M-cones, covering 1×1 -mm² areas at the midperipheral region of the superior wing of the retina. The micrographs were used to compose collages using Photoshop.

Each nucleus of the immunolabeled M-cones was visualized using the zoom tool (Supplementary Figs. S1, S2) and each nucleus was marked with a white dot using the paint tool in Photoshop. The circular dots were slightly lesser in size of the actual nuclei and were kept even throughout the entire working space. This way, in cases when two nuclei are close to one another, the two dots marking them neither touched each other nor overlapped. The resulting “nuclei-positions map” allowed easy identification of the position of each M-cone in the micrographed retinal area. Also, using these images, the density of M-cones (total number/ 1×1 m², $n = 3$ –5 animals for each group) was measured.

Statistical Analysis

The previously described nuclei-positions maps were used for the NND and Voronoi analyses. For the Voronoi analysis, the Voronoi domain for each cell was generated and the areas of each polygon were calculated and plotted in a histogram. For the NND analysis, the distance to the nearest neighboring cell was measured for each dot.⁴³ The distributions were plotted in a histogram. In turn, for the Voronoi analysis, the Voronoi domain for each cell was generated and the areas of each polygon were calculated and plotted in a histogram. To remove the artifacts induced by the edge, we did not include cells around the boundaries.

These NND histograms were then compared with simulation distributions generated from a random-positions model. This model was programmed to yield expected distributions for mosaics that were random in the spacing of cells. The model took into account the constraint in spacing induced by the cone-nucleus size (~ 5 µm). The importance of such a constraint has been discussed at length in a recent review.⁴⁴ Without this constraint, the theoretical distribution rises slower to the peak than predicted by the constrained model.⁴³

The curves generated by this model were overlaid on the NND histograms for comparison.

We also extracted statistics from the distributions for analysis. The skewness of the Voronoi distribution also was determined. The formula used for quantifying skewness was:

$$g_1 = \frac{\frac{1}{n} \sum_{i=1}^n (x_i - \bar{x})^3}{\left(\frac{1}{n} \sum_{i=1}^n (x_i - \bar{x})^2 \right)^{3/2}},$$

where x_i is the area of the i^{th} Voronoi domain and $\bar{x}$ is the sample mean. Also, the coefficient of clustering (CC) is determined by the ratio between the global coefficient of variation and the average local coefficient of variation in Voronoi domain sizes. The formula is as follows:

$$c = \frac{n\bar{x}}{\sigma_x \sum_{i=1}^n \frac{\bar{a}_i}{\sigma_{a_i}}},$$

where σ_x is the standard deviation of all the Voronoi domains, $\bar{a}_i$ and σ_{a_i} is the mean and SD of size of neighboring Voronoi domains of i^{th} domain, respectively. All the statistics were expressed as mean $\pm$ SEM. Two-way unbalanced ANOVA and post hoc Tukey's least-significant difference procedure were used to examine the difference among a group of means. The tests were performed and graphs were generated by MATLAB version 7.4.0 (The MathWorks, Inc., Natick, MA, USA). A difference between the means of separate experimental conditions was considered statistically significant at α level of 0.05.

RESULTS

Absence of Glial Activation and M-Opin Cone Cell Death With TIMP-1

First, the safety of TIMP-1 in concentration and volume used for intraocular injections in this study (25 µg/mL, 4 µL) was tested. To check if TIMP-1 was toxic to retinal cells, normal retinas from the control and the TIMP-1-treated groups were immunostained with GFAP, a marker for glial activation associated with retinal degeneration.^{45,46} The controls showed no significant upregulation of GFAP expression at 1 hour (data not shown), 2 weeks (Fig. 1A), and 6 weeks (data not shown). The GFAP expression is seen predominantly in the nerve fiber layer (NFL). Similar results were observed among the TIMP-1 groups; that is, no significant upregulation of GFAP at 1 hour (Fig. 1B), 2 weeks (Fig. 1C), and 6 weeks (Fig. 1D). Moreover, we did not observe TUNEL-positive cells in all groups (data not shown). In summary, TIMP-1 did not cause glial activation and cell death in both normal and RP retinas.

In addition, the number of M-cones was measured within the 1×1 -mm² areas at the midperipheral region of the superior wing of the retinas. Retinas of all four conditions showed decreasing mean M-cone densities with age and increasing survival period (Fig. 1E; $P < 0.000001$, two-way ANOVA). This is a common observation that arises with the aging of animals and the subsequent retinal growth.^{11,47,48} However, no statistically significant differences were observed in the number of M-cones between the control and the TIMP-1 groups for both normal and RP retinas ($P = 0.5576$, two-way ANOVA). The greatest visible difference in the mean M-cone density occurred in RP retinas 6 weeks after TIMP-1 application ($P < 0.05$).

FIGURE 1. Confocal micrographs taken from cryostat sections of normal retinas processed for GFAP immunoreactivity shown for the 2-week control (A), and the 1-hour (B), 2-week (C), and 6-week (D) TIMP-1 groups. The drug caused no significant upregulation of GFAP expression. The summary graphs illustrated for mean cone density (E) measured from the $1 \times 1\text{-mm}^2$ sampling areas (in the superior midperipheral region) of all normal control, TIMP-1-treated normal, RP control, and TIMP-1 RP retina groups ($n = 3\text{--}5$ animals per group). Data are presented as mean $\pm$ SE. GCL, ganglion-cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer-plexiform layer. Scale bar: 50 μm .

Disturbance of the Mosaic of M-Cones in RP Retinas With TIMP-1

To examine if exogenous application of TIMP-1 can modulate the M-cone mosaic in vivo, this drug was administrated intraocularly into RP rat eyes. The M-cones were labeled in

the whole-mount retinas in all groups. The RP retinas of the controls (Figs. 2A–C) and the TIMP-1-treated groups (Figs. 2G–I) immunostained with M-opsin showed fairly intact cone morphologies. For mosaic quantification, we used the nuclei-positions map (Figs. 2D–F, 2J–L). In these figures, the geometry of their mosaic can be seen clearly. The control RP retinas

FIGURE 2. Confocal micrographs taken from whole-mount RP retinas processed for M-opsin immunoreactivity (A–C, G–I) and nuclei-position maps (D–F, J–L). In these maps, each *dot* represents a nucleus of an M-cone as obtained from the micrographs. The micrographs for control groups show P45 RP (A), P59 RP (B), and P87 RP (C) retinas 1 hour, 2 weeks, and 6 weeks after saline application, respectively. Rings are observed in the mosaics of RP controls (A–F). The micrographs for TIMP-1 groups show P45 RP TIMP-1 (G), P59 RP TIMP-1 (H), and P87 RP TIMP-1 (I) retinas 1 hour, 2 weeks, and 6 weeks after application of the drug, respectively. The TIMP-1 loosens rings and increases the homogeneity of the mosaic of M-cones (G–L). 1HR, hour. Scale bars: 500 μ m.

FIGURE 3. Histograms generated from the Voronoi analysis on the $1 \times 1\text{-mm}^2$ sampling areas from all RP controls (A–C), TIMP-1-treated RP (D–F), and normal controls (G–I) ($n = 3$ –5 animals per group). Results are shown with survival times of 1 hour, 2 weeks, and 6 weeks. Examples ($\sim 170 \times 170 \mu\text{m}$) of the resulting Voronoi domains are shown for each group. The summary graphs for the mean skewness values obtained from the Voronoi domain distribution curves are plotted for each group (J). Also, the graph for the mean CC measures in all groups is illustrated (K). Data are presented as mean $\pm$ SE. * $P < 0.05$.

showed nuclei forming the rim of the rings and the cones' processes pointing toward the center of the regions devoid of cell bodies (Figs. 2A–C). Furthermore, the size of these rings increased with age (Figs. 2D–F), which was consistent with our previous observations.¹¹ Such M-cones mosaic showed remarkable change with TIMP-1. The rings lost first their sharpness and eventually disappeared (Figs. 2J–L). Even after only 1 hour, the rings became less defined and smaller compared with the

control group (Fig. 2J). At 2 weeks, the rings disappeared and cones redistributed themselves homogeneously (Fig. 2K). Such striking change continued even at 6 weeks (Fig. 2L).

Voronoi analysis on RP retinas was performed to quantify changes in homogeneity of the mosaic and the gradual disappearance of rings. Examples of the resulting Voronoi tessellation are shown in insets beside the histograms (Figs. 3A–F). In the RP-control retinas, most Voronoi domains were

small, as M-cones are clustered around the rings. Furthermore, a few large Voronoi domain areas were observed. These larger areas resulted from the regions with few or no cones in the rings. Hence, the histograms from the data had longer tails, resulting in highly skewed distributions (Figs. 3A–C, 3J). The insets in Figures 3A through 3C illustrate the alternation between small and large Voronoi domains in the RP retinas.

The alternation between small and large Voronoi domains is apparently not random in RP retinas, but appears to show a specific pattern in that small domains are surrounded by other small domains, whereas large domains are surrounded by other large domains (Figs. 3A–C). We quantified this correlation between the sizes of neighbor domains by calculating the CC. The CC is the ratio between the global coefficient of variation and the average local coefficient of variation in Voronoi domain sizes. If the correlation did not exist, then the large and small Voronoi domains would be equally likely everywhere, causing the local and global coefficients of variation to be similar. Consequently, the CC would be near 1. If instead, the large domains were near each other and the small domains were close to other small domains, then the local coefficient of variation would be small because of the similarity in neighborhood statistics. However, the global coefficient of variation would be large, since one would have both large and small domains across space. Thus, the CC would be larger than 1. Hence, the CC emphasizes a property not clarified by the Voronoi domain histograms. Their skewness shows the existence of multiple small domains and few large domains, but does not show that these domains exhibit spatial segregation. In turn, the CC can show this segregation. Therefore, the CC highlights that large domains occur only in holes, whereas small domains occur only in the rims of the rings. Only when the CC is greater than 1 do we have statistical evidence of the segregation.

As the experimental data showed, RP retinas exhibited high CC (Fig. 3K), confirming that the spatial alternation between small and large Voronoi domains was not random. In contrast, in TIMP-1-treated RP groups, the rings gradually disappeared and cones redistributed themselves homogeneously. With increasing survival periods, the cones spread out to occupy areas inside rings, and large Voronoi domains became smaller, and less skewed (Figs. 3D–F, 3J). Voronoi analysis on normal control retinas (Figs. 3G–I) was performed to compare the homogeneity of the mosaic between TIMP-1-treated RP groups and normal control groups. Examples of the resulting Voronoi tessellation are shown in insets beside the histograms (Figs. 3G–I). In the normal control retinas, the distribution of Voronoi domains was close to Gaussian, thus less skewed (Figs. 3G–I, 3J). To compare the distribution of Voronoi domains among three groups (RP control, RP TIMP-1, and normal control), we examined both skewness of the distributions and their CC. The skewness of the distributions was significantly different from RP-control and TIMP-1-treated RP and normal control retinas ($P < 0.0001$, two-way ANOVA). Post hoc analysis showed significantly lower skewness value in normal control groups and RP TIMP-1 groups compared with RP controls at both 2 weeks and 6 weeks (post hoc test, $\alpha = 0.05$). This indicated that Voronoi domains with extremely larger size are reduced, and cones in RP retinas became more homogeneous with TIMP-1 after 2 weeks. Furthermore, homogeneity of cone mosaic is restored closely to normal control groups after 2 weeks. This was also confirmed by the measurement of CC. Our results showed statistically significant differences in CC between control RP and TIMP-1-treated RP groups with 2 weeks or more of treatment (Fig. 3K, $P = 0.0001$, two-way ANOVA). The M-cones in TIMP-1-treated RP retinas were still highly clustered at 1 hour drug exposure; however, the mosaics became significantly closer to normal after 2

weeks (post hoc test, $\alpha = 0.05$). In summary, TIMP-1 induced mosaics of M-cones in RP retinas to gain homogeneity and become close to normal.

Tissue Inhibitor of Metalloproteinase-1 Injection Induces Irregularity of M-Opsin Cones in RP Retinas

We examined if the homogeneous M-cone mosaics in TIMP-1-treated RP retinas are also regular, as in normal mammalian retinas.^{11,12} Two critical hallmarks for a normal cone mosaic are homogeneity and regularity. Homogeneity means that the spatial statistics of cones are similar in different regions. In turn, regularity means that the distance from a cone to its neighbors is similar for different cones. In Figure 3, we showed that TIMP-1 induced mosaics of M-cones in RP retinas to gain homogeneity. Next, we performed NND regularity index (NND-RI) to determine the regularity. Thus, we measure regularity by the RI.⁴³ It is the ratio of the mean to the SD of the distances from each cell to its nearest neighbor. In addition, we plotted a distribution of the NND for the random-position model with the same density and with minimum distance of 5 μm at each time point (solid lines). The histograms for the normal control groups showed near-Gaussian distributions that did not conform well to the predictions from the random-positions model (Figs. 4A, 4B). The mean NNDs in the normal control retinas at 2 weeks and 6 weeks were $10.29 \pm 0.08 \mu\text{m}$ and $10.88 \pm 0.07 \mu\text{m}$, respectively. These distributions were distinct from the random-positions model. Subsequently, the mosaics showed high regularity with RI value of 3.94 ± 0.03 and 4.22 ± 0.26 at 2 weeks and 6 weeks, respectively. However, the NND distribution changed with TIMP-1-treated RP groups. The distributions from the TIMP-1-treated RP retinas showed smaller mean NNDs of $8.95 \pm 0.04 \mu\text{m}$ and $9.15 \pm 0.31 \mu\text{m}$ at 2 weeks and 6 weeks, respectively (Figs. 4C, 4D). The RI values at 2 weeks and 6 weeks were $3.31 \pm 0.12 \mu\text{m}$ and $3.08 \pm 0.14 \mu\text{m}$, respectively.

To understand if lower RI values of M-cone mosaic in TIMP-1-treated RP retinas were a direct consequence of TIMP-1 treatment or if they were independent of TIMP-1 effect, we examined the regularity also in normal retinas treated with TIMP-1 (Figs. 4E–H). To address this question, we applied TIMP-1 to normal retina that has both homogeneity and regularity. The M-cones were labeled in the whole-mount retinas in all groups (control groups: Supplementary Figs. S1A–C; TIMP-1: Supplementary Figs. S1G–I). The images of marked nuclei of M-cones help visualize the geometry of their mosaics (Supplementary Figs. S1D–F, S1J–L). The M-cones in control groups showed regular and homogeneous distribution patterns (Supplementary Figs. S1A–C) that were similar to those seen in the normal mammalian retinas.^{11,12} The nuclei-positions map emphasizes this similarity in M-cone patterns (Supplementary Figs. S1D–F, S2); however, the mosaic of M-cones showed some changes with TIMP-1 (Supplementary Figs. S1G–I, S2). First, the orientation of array of the outer segments was disturbed in some regions (Supplementary Figs. S1G–I, squares). Rather than showing steady orientation as in control groups, variable orientations were sometimes observed in retinas with TIMP-1 (Supplementary Figs. S1G–I, squares). More importantly, TIMP-1 led to change in the arrangement of some cell bodies after 2 weeks that seem to show loss in regularity (Supplementary Figs. S1K, S1L, ellipses; although not much after 1 hour, Supplementary Fig. S1J).

The NND analysis on TIMP-1-treated normal retinas showed that the distribution became more skewed and broader compared with normal controls with significantly less mean NND of $9.93 \pm 0.21 \mu\text{m}$ by 6 weeks (Figs. 4E, 4F). The

FIGURE 4. Distribution of distances between nearest-neighbor M-cones within the $1 \times 1\text{-mm}^2$ sampling areas from normal control (A, B), TIMP-1-treated RP (C, D), and TIMP-1-treated normal groups (E, F) ($n = 3\text{--}5$ animals per group) at 2 weeks and 6 weeks after treatments. The histograms are overlaid with distributions generated from the random-positions model (solid line in each histogram). With the application of TIMP-1, the NND distributions became closer to the simulated random distribution. The summary graphs for mean NND (G), and the mean RI (H) for all groups are illustrated. Data are presented as mean $\pm$ SE. $*P < 0.05$.

mean RI also declined compared with normal controls, with value of $3.19 \pm 0.16 \mu\text{m}$. In addition, the NND distribution showed better fit to the random distribution (solid lines). We then compared the mean NND (Fig. 4G) and RI (Fig. 4H) for normal control, RP, and normal retinas with TIMP-1 treatment. The two-way ANOVA analysis showed significant differences in both mean NNDs and RIs among the different groups of retinas (Fig. 4G mean NND, $P = 0.0001$; Fig. 4H RI, $P = 0.0005$), but not between different stages (2 weeks and 6 weeks) after intraocular treatment. Compared with the normal control retinas, the TIMP-1-treated normal retinas showed statistically lower mean NND and RI at 6 weeks. (Figs. 4G, 4H, post hoc test, $\alpha = 0.05$). However, the mean NND in TIMP-1-treated normal retinas were still significantly higher than in TIMP-1-treated RP retinas (Fig. 4G, post hoc test, $\alpha = 0.05$). Consistent with this observation, the mean RIs in TIMP-1-treated normal retinas were lower than normal controls; however, not significantly different from that of the TIMP-1-treated RPs (Fig. 4H, post hoc test, $\alpha = 0.05$). These indicated that M-cone mosaic in TIMP-1-treated RP retinas did not reach the degree of regularity seen in normal retinal mosaics. In addition, TIMP-1 led to loss of local spatial regularity in the mosaics of M-cones in normal rat retinas. In summary, the loss of regularity in TIMP-1-treated RP retinas may largely be caused by TIMP-1.

Remodeling of Müller Cell Processes in RP Retinas With TIMP-1

In this article, we focused on TIMP-1 because it is one of the regulators of the ECM, thus being important for cellular migration. Another retinal process contributing to the migration of neurons is the Müller glial cell. We thus decided to test whether Müller cell processes in RP retinas were also affected by TIMP-1. Therefore, we immunostained RP-control and TIMP-1-treated retinas with M-opsin and GS, a marker for Müller cells.^{49,50} Consistent with our previous work,¹² the RP-control retina showed remodeled processes of the Müller cells filling the insides of each ring of M-cones after 1 hour (data not shown), 2 weeks (Fig. 5A), and 6 weeks (data not shown). A high-magnification view of a ring marked by the inset rectangle revealed these remodeled processes more closely (Fig. 5B). The RP retinas at 1 hour after application of TIMP-1 showed disturbance of rings as they became smaller and less distinct (Fig. 5C). A higher-power micrograph revealed that the Müller cell processes were filling inside the center of the shrinking rings (Fig. 5D). The RP retinas at 2 weeks (Figs. 5E, 5F) and 6 weeks (data not shown) after application of TIMP-1 showed homogeneously distributed M-cones and Müller-cell processes. In summary, these results indicated that the Müller-cell processes in RP retinas are also remodeled with cone mosaic significantly on application of TIMP-1.

DISCUSSION

Tissue Inhibitor of Metalloproteinase-1 Does Not Cause Cell Death

Why does TIMP-1 treatment cause such dramatic effects in RP retinas? The results reveal that this drug is not acting through retinal damage. To start, neither saline nor TIMP-1 introduce reduction in the cone density (Fig. 1). Moreover, the glial activation associated with TIMP-1^{45,46} is also not detected in normal retinas (Fig. 1), and lack of significant TUNEL-positive staining indicates no sign of cell deaths in these retinas (results not shown). Thus, the reduction of the mean cone density that we observe with greater survival time is not explained by cell deaths but by the growth of the total retinal area with age (Fig.

1). In addition, the density is the number of cells divided by area. Hence, any density changes must be due to area variations. Furthermore, we also demonstrated previously that the mean retinal areas from P30 to P180 increased significantly in normal and RP retinas.¹¹ Therefore, the retinas were shown to grow with age. Such growth results in the declining density of different types of retinal cells.^{11,47,48} In particular, greater retinal expansion in the peripheral retinal regions compared with the central region^{51,52} may have made our midperipheral regional density results more significant.

Mosaics of M-Cones Can Be Manipulated by TIMP-1 Treatment

In the present study, two mosaic properties were studied statistically: homogeneity and regularity. Both properties are important, as they are the basis of even sampling of visual world, which provides visual acuity.^{9,10} One of the main results of the current study is that TIMP-1 causes change in the mosaic of cone photoreceptors in RP retina to become more homogeneous. Homogeneity is a measurement of the spatial statistical properties of the mosaic and is as constant as possible over large portions of the retina. When a mosaic exhibits rings, the mosaic is not homogeneous, because the statistics in their rims are different from those in the areas with little or no cones (center of rings). Therefore, we are looking for an analysis that will provide the degree of global homogeneity and existence of holes. Classical tools, such as quadrat analysis, would provide only the former. In turn, with largest-empty-space analysis, only information about existence of holes is provided. In contrast, the Voronoi domain analysis, although not typically used as a homogeneity test, can detect the global homogeneity and existence of holes (Figs. 3B, 3E). Thus, to emphasize ring-induced inhomogeneity, we measured the distribution of areas of Voronoi domains. These domains are large inside the rings and small in their rims. Such rings become visibly disturbed and less distinct after only 1 hour of TIMP-1 treatment (Figs. 2G, 2J). By 2 weeks, the rings are no longer obvious, as cells cover the space homogeneously (Figs. 2H, 2K). The Voronoi domain analysis results statistically confirmed such observation. The skewness of the small Voronoi domain areas in RP retinas declined significantly as M-cones start to migrate to fill inside the empty rings with TIMP-1 treatment (Figs. 3D–F, 3J). Moreover, as the cells move away from the crowded rim of rings, the mean CC decreases significantly over time. All these changes that TIMP-1 brings to the retina make the mosaic properties closer to what is observed in the normal retinas (Figs. 3G–K).

Another important result from our study is that the regularity of the mosaic is lost with TIMP-1 treatment. We think of regularity as an even or uniform arrangement at small spatial scales (i.e., relatively local). One can measure regularity in many ways, but in this article, we used the simplest definition; namely, the similarity of distances between nearest neighbors. The results from the NND analysis showed that TIMP-1 induced mosaic to become closer to a random distribution with significantly less NND and RI compared with the normal retinas (Figs. 4A–D, 4G, 4H). Thus, although clear improvement of homogeneity is achieved, the mosaic became irregular.

Ultimately, the aim of drug treatment therapy is to improve both homogeneity and regularity. However, with TIMP-1 treatment, we see a clear improvement of homogeneity without accompanying restoration of regularity. Thus, to better understand if such irregularity is a direct consequence of TIMP-1 treatment or it is independent of TIMP-1 effect, we applied the treatment to normal retinas that have homoge-

FIGURE 5. Confocal micrographs taken from RP whole mounts of control and TIMP-1 groups processed for GS (green) and M-opsin (red) immunoreactivities. Double exposure of control retina at 2 weeks (A) and its higher-power micrograph (B) show rings of M-cones around remodeled Müller-cell processes in characteristic broccoli-like shape. Just 1 hour after application of TIMP-1, M-cones and Müller-cell processes begin losing their broccoli-like shapes (C). A higher-power micrograph shows this loss more clearly (D). After 2 weeks, the mosaic of M-cones and Müller-cell processes is almost homogeneous (E). However, a higher magnification reveals some tendency for some groups of M-cones to migrate closer to each other, showing that the mosaic is becoming less regular (F). Scale bars: 100 μ m.

neous and regular mosaic. As results, we observed the M-cone mosaic significantly loses its regularity at 6 weeks and becomes close to a random distribution. Thus, the loss of regularity may largely be caused by TIMP-1. Even if TIMP-1 fails to promote regularity, the effects of this drug on homogeneity appear to be so dramatic that we may still consider TIMP-1 as a potential therapeutic tool. The TIMP-1 would improve sampling of the visual field simply by causing homogeneity.

A possible reason for dystrophic retinas to show more dramatic change in the mosaic pattern with TIMP-1 may be that there is more space for cones to migrate after the rods

die.¹³ In our previous study, death of rods induces slow rearrangement of cones into regular mosaics of rings. Although the number of cones remains similar in normal and dystrophic retinas even at an older age, rods in RP die in “hot spots” that increase progressively as circular waves, leaving behind “rodless” zones.^{11,13} Our work also clearly demonstrated that Müller cell processes remodel to occupy these zones, interact with the cones, and induce cone migration to the edges of the holes of rods.^{11,12} Therefore, dramatic change in the mosaic with TIMP-1 may result in more space for cones to migrate.

What Are the Possible Mechanisms Underlying Modulation of Mosaics of M-Cones With TIMP-1?

The simplest hypothesis is that TIMP-1 acts through the ECM. For cones to migrate during the change in the mosaic, interactions between the cells and the ECM are necessary.^{53,54} Cell migration depends on the stiffness of the ECM.⁵⁵ As the ECM comprises mostly collagen, the mammalian enzymes that modulate its functions are the MMPs and their inhibitors, the TIMPs.^{56,57} For MMP and TIMP to play an essential role in the organization of the ECM,^{23,24} the balance in the level of these enzymes is crucial. Such balance is disrupted in pathological retinas, and often, the level of TIMP-1 is significantly upregulated with ocular disease.^{34–36} Tissue inhibitor of metalloproteinase-3 mRNA was also overexpressed in human RP and Sorsby's fundus dystrophy conditions and was localized to structures including photoreceptor inner segments and Bruch's membrane.^{58–60} Thus, a reasonable hypothesis to explain our results is that exogenous application of TIMP-1 disturbed the enzymatic balance and the mechanical properties of the ECM even further.²¹ There is also evidence that TIMP-1 can affect the migration of cells by modulating focal adhesions composed of integrins and various cytoplasmic proteins in the ECM, which cells use as aids during their migration.¹⁸ Thus, if controlled release of growth factors from the ECM is necessary to maintain the M-cones in rings, the fall in the release of growth factors sequestered in the ECM due to addition of TIMP-1 would lead to fewer M-cones in rings.

Finally, it is well known that glial cells are also regulated by signals from the ECM.⁶¹ The above-mentioned integrins are found expressed in glia and modulate their migration and organization.⁶¹ Our hypothesis is that Müller glial cells are involved in the migration of cones by complex interactions with surrounding ECM. Müller cell processes are shown in close association with migrating cones (Fig. 5).¹² In a previous study,¹² we showed effects of DL- α -aminoadipic acid (AAA) on cone mosaics, which were similar to those observed in our current study with TIMP-1. The AAA is a gliotoxin, which disrupts Müller cell metabolism.^{62–65} Thus, TIMP-1 may have affected the interaction between cones and Müller cells through ECM. Further support for this hypothesis comes from Müller cells playing a role in the regulation of expression and production of TIMP and MMP through a feedback system involving the ECM.^{66,67} Our results are consistent with this hypothesis, as Müller-cell processes remodel with cones in TIMP-1-treated RP retina (Fig. 5).

CONCLUSIONS

We have shown that exogenous application of TIMP-1 can significantly modulate the mosaics and restore its homogeneity in the RP retinas. The resulting mosaic also loses regularity and becomes close to random distribution both in the RP and in normal retinas. All these changes occur without being toxic to retinal cells. Our findings have clear therapeutic implication, as they suggest that treatment with TIMP-1 could improve sampling of visual field by improving homogeneity. In the future, we will assess the efficacy of TIMP-1 when administered before the appearance of rings and later stages of disease.

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