**TIMP-1 Affects the Spatial Distribution of Dendritic Processes of Second-Order
Neurons in a Rat Model of Retinitis Pigmentosa**

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Key words

Retinitis pigmentosa; tissue inhibitor of metalloproteinase-1(TIMP-1); cone mosaic; bipolar cell; horizontal cell, synaptic protein; cone outer segment

Abstract

Retinitis Pigmentosa (RP) is an inherited disorder that may lead to blindness. In the rhodopsin S334ter-line-3 rat model of RP, the death of rods induces spatial rearrangement of cones into regular ring mosaics. Using this model, we discovered that the ring mosaics are restored to a homogeneous distribution upon application of tissue inhibitor of metalloproteinase-1 (TIMP-1). In this study, we further investigated the cone migration and spatial distribution of second-order neurons and their connections to cones in the presence or absence of TIMP-1 using immunohistochemistry to identify retinal neurons and their connections with cones. M-opsin cell bodies and their outer segments were evaluated to determine whether TIMP-1 delays the degeneration of outer segments of cones. We observed that during cone rearrangement into ring mosaics in RP retina, dendritic processes of second-order neurons undergo remodeling to maintain their synaptic connections with the cones in the rings. TIMP-1 treatment induced the cones to rearrange and dendritic processes of second-order neurons to return to a more homogeneous spatial distribution. In addition, TIMP-1 treatment protected the outer segments of cones at later stages of retinal degeneration. Our findings clearly demonstrate that despite their dramatic spatial rearrangement, cones and second-order neuron processes maintain their synaptic connections before and after TIMP-1 treatment.

1. Introduction

Retinitis Pigmentosa (RP) is a leading cause of inherited blindness that results from one of many possible gene mutations (Farber and Lolley 1974; Bowes et al. 1990; Rosenfeld et al. 1992; Marc et al. 2003). Rod degeneration in RP frequently results in the death of cones, although the extent of cone degeneration can vary among patients (Ripps 2002; Delyfer et al. 2004; Hartong et al. 2006) and animal models (Carter-Dawson et al. 1978; LaVail et al. 1997). Because cones are mainly responsible for day and high-acuity vision, their loss leads to a reduction in the quality of life and eventually, to vision loss. Thus, understanding the molecular and cellular components that maintain cone viability and the appropriate synaptic connections to second-order neurons in degenerative animal models are relevant issues to explore for potential therapeutic strategies such as gene therapies (Vollrath et al. 2001; Jacobson et al. 2005; Beltran et al. 2012; Lheriteau et al. 2014) and stem cell transplantation (Kim et al. 2005; Siqueira et al. 2011).

Previous studies have provided data on the rewiring of retinal neurons and the timing of retinal plasticity after rod death in vertical sections of S334ter-line-3, Royal College of Surgeons, P23H-line1 rat retinas and *rd1* mouse retina (Strettoi et al. 2003; Cuenca et al. 2004; Cuenca et al. 2005; Ray et al. 2010; Martinez-Navarrete et al. 2011). In the whole-mount retinas of S334ter-line-3 rat, a transgenic model developed to express an early termination stop codon thus creating a truncated rhodopsin mutation similar to a human form of RP, rods die in “hot spots” and Müller cell processes remodel to occupy these zones and interact with cones, causing them to form orderly mosaic of rings (Lee et al. 2011; Ji et al. 2012; Zhu et al. 2013). This appearance of dark patches or “holes of photoreceptors” was also reported in cyclin D1 (*cd1*) mutant and P23H-line1 rat (Ma et al. 1998; Garcia-Ayuso et al. 2013). Furthermore, the dark patches were noted in several human eye diseases caused by retinal dystrophy, inherited retinal degeneration, and photo-pigment genetic perturbations in

M-opsin cones (Carroll et al. 2004; Choi et al. 2006; Duncan et al. 2007; Rossi et al. 2011). The center of these rings lack photoreceptors, indicating local loss of vision. Recently, we found that the shape of the receptive field of RGCs changed in RP retina. The structure was reminiscent of that observed in the cone mosaic (Yu WQ, et al. IOVS 2014; 55: ARVO E-Abstract 6198). Taken together, we hypothesized that the dendritic processes of second-order neurons undergo remodeling to maintain their connections with the remodeled cones in rings.

In a recent report, we discovered that treatment with tissue inhibitor of metalloproteinase-1 (TIMP-1), one of the key regulators of the extracellular matrix (ECM) critical for cellular migration, induces statistically significant changes to the cone mosaic in S334ter-line-3 retina (Ji et al. 2015). Treatment with TIMP-1 in RP retina allows cones to assume a similar homogeneous distribution to that in normal retinas (Ji et al. 2015). In this study, we further investigated in this rat model the impact of TIMP-1 on the spatial distribution of the remodeled processes of second-order neurons and their connection with cones.

2. Materials and Methods

2.1. Animals

The third line of albino Sprague-Dawley (SD) rats homozygous for the truncated murine opsin gene (created a stop codon at Serine residue 334; S334ter-line-3) was obtained from Matthew LaVail, Ph.D. (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. This model shall be referred to as the RP model in the rest of the article. For control, age-matched SD rats (Harlan, Indianapolis, IN, USA) were used. Both sexes of normal and S334ter-line-3 rats were used. All rats were housed under cyclic 12/12-hour light/dark conditions with free access to food and water. 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.

2.2. Administration of TIMP-1

The procedure on preparation of TIMP-1 was identical with our previous study (Ji et al. 2015). At postnatal days (P) 45, 4 μl of 25 $\mu\text{g/ml}$ TIMP-1 (Sigma-Aldrich Corp., St. Louis, MO, USA) was administered by intravitreal injection with a Hamilton syringe (Sigma-Aldrich Corp) through the sclera at the level of the temporal peripheral retina. For control animals, 4 μl of the sterile saline was injected. Surgeries on rats were performed under anesthesia induced by intraperitoneal injection of ketamine (20 mg/ kg; KETASET, Fort Dodge, IA, USA) and xylazine (5 mg/kg, X-Ject SA; Butler, Dublin, OH, USA).

2.3. Tissue Preparation

Normal SD rat at P60 (n=5), homozygous S334ter-3 rats at P60 (n=10) and P90 (n=3) and TIMP-1 injected homozygous S334ter-3 rats P60 (n=6) and P90 (n=3) were used. Animals were deeply anesthetized by intraperitoneal injection of Euthasol (40mg/kg, Fort Worth, TX, USA) and the eyes were enucleated. Animals were then euthanized with an overdose of Euthasol. 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 90 minutes at 4°C. Following fixation, the eyecups were transferred to 30% sucrose in PB for 24 hours at 4°C. For storage, all eyecups 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 Leica cryostat at a thickness of 20 μ m. For whole mount, the retinas were carefully isolated from the eyecups and dissected as whole-mounts.

2.4. *Immunohistochemistry*

For vertical section immunohistochemical staining, 20 μ m thick cryostat sections were incubated in 10% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or 10% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Sections were then incubated overnight with primary antibodies: rabbit polyclonal antibody directed against green opsin (M-opsin, Zhu et al. 2013, dilution 1 : 2,000); protein kinase C α (PKC α ; Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1 : 1,000); calbindin (Millipore, Temecula, CA, USA, dilution 1 :

1,000); mouse monoclonal antibody directed against Go α (Chemicon, Temecula, CA, USA, dilution 1 : 1,000); synaptophysin (Sigma-Aldrich Corp., dilution 1 : 10,000); bassoon (Stressgen, Ann Arbor, MI, USA, dilution 1: 5,000); Guinea pig polyclonal antibody directed against vesicular glutamate transporter 1 (VGLUT1; Chemicon, dilution 1 : 5,000). Retinas were washed in PBS for 45 minutes (3X15 minutes) and afterward incubated for 2 hours at room temperature in either carboxymethylindocyanine (Cy3)-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, dilution 1:500) or Alexa 488-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene, OR, USA; dilution 1:300). For double-label studies, the sections were incubated overnight in a mixture of primary antibodies and 2 hours in a mixture of corresponding secondary antibodies. Next, the sections were washed for 30 minutes with 0.1M PB and cover slipped with Vectashield mounting medium (Vector Labs, Burlingame, CA, USA).

For whole-mount immunohistochemical staining, the same procedures described above were used. However, for enhanced antibody penetration, tissues were treated with 1% Triton X-100 in 0.1M PBS before NGS or NDS, and primary antibodies were diluted in 0.5% Triton X-100 in 0.1M PBS. Incubation times with the primary antibodies were longer (48 hours) and so were those with the secondary antibodies (24 hours). For double-label and triple-label studies, whole mounts were incubated for 48 hours in a mixture of primary antibodies in 0.5% Triton X-100 at 4°C. After this incubation, whole mounts were rinsed for 30 minutes with 0.1 M PBS. Afterward, they were incubated with mixture of corresponding secondary antibodies for overnight at 4°C. The secondary antibodies used in triple-label were Cy3-conjugated affinity-purified donkey anti-guinea pig IgG (Jackson Immuno Laboratories; dilution 1:500), Cy5-conjugated donkey anti-mouse IgG (Jackson Immuno Laboratories; dilution 1:50) and Alexa 488-conjugated anti-rabbit IgG (Molecular Probes; dilution 1:300).

Whole mounts were then washed again for 30 minutes with 0.1 M PB and cover slipped with Vectashield mounting medium.

2.5. *Image analysis*

Sections and whole mounts were 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, San Jose, CA, USA). All Photoshop adjustments were carried out equally across sections.

2.6. *Cone cell counts*

The density of nuclei and corresponding outer segment was counted in three retinal whole-mount preparations from each group. Confocal micrographs of the whole-mounted retinas (n=3, animals per group) were taken at the focal level near cell bodies of immunoreactive stained M-opsin cones, covering a 1 x 1 mm² area at both the superior and inferior regions (1mm away from optic disc) of the retina. At these locations, we made serial optical sections using a confocal microscope. By following each immunological stained M-opsin cone throughout the sections, we ensured that every M-opsin cell body with its outer segment in the selected region was counted.

2.7. *Statistical analysis*

All the statistics were expressed as mean $\pm$ standard errors of the mean. Two-way ANOVA with post-hoc Tukey's least significant difference procedure (LSD test) were used to examine the differences among the groups. The analysis was performed and graphs were generated by

GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). A difference between the groups was considered statistically significant at p value of 0.05.

3. Results

3.1. Remodeling of the dendritic processes of second-order neurons in RP

Consistent with our previous work, we observed homogenous distribution of M-opsin cones in normal retina at P60 (Fig. 1A). In contrast at P60, cones in RP retinas had migrated to form ring-like patterns, leaving large holes with few or no cones (Fig. 1B). Finally, cones at P60 in all the TIMP-1 treated RP retinas had rearranged themselves into a homogeneous pattern (Fig. 1C). Together, these results strongly suggest that the cone reorganization driven by rod death in RP can be restored by TIMP-1 treatment (Ji et al. 2015).

We investigated if the rearrangement of cones into rings affects spatial distribution of second-order neurons and their dendritic processes. For this experiment, we performed immunohistochemistry on vertical sections and whole-mounts of P60 normal and RP retinas. Vertical sections and whole-mount retinas were immunologically stained with specific antibodies to identify M-opsin cones (Fig. 2A, B, E-H), rod bipolar cells (PKC α , Fig. 2C, D, I-L), ON bipolar cells (Go α , Fig. 3A, B, E-H), and horizontal cells (Calbindin, Fig. 3C, D, I-L) (Haverkamp and Wässle 2000). The images were taken from the mid-peripheral part of the superior (3 mm away from the optic disc) of P60 vertical and whole-mount retinas. All the pictures of whole-mount retina were taken in the dotted lines at the corresponding vertical sections (Figs. 2 and 3).

In normal retina, immunoreactive staining with the antibody for M-opsin revealed cones that were vertically aligned (Fig. 2A) and homogeneously organized with uniform spacing across the retina (Fig. 2B). In RP retina, three different optical levels (marked with dotted lines in the vertical sections, Fig. 2E, I and Fig. 3E, I) were examined to determine the spatial distribution of M-opsin cones, rod bipolar cells, ON-bipolar cells, and horizontal cells (Figs. 2 and 3). In RP retinas, M-opsin immunoreactive staining revealed that cones lay flat

against the outer retina (Fig. 2E), cone outer segments (COS) were also shorter and distorted (Fig. 2E, arrow). We also observed separate regions full of clusters of cell bodies followed by regions devoid of cell bodies but richer in long processes (Fig. 2E, arrowhead) (Ji et al. 2012). Furthermore, some processes of cones were noted near the cell bodies (Fig. 2E, open arrowhead). The whole-mount showed M-opsin cones that were distributed outside the rim of the ring project their processes close to their cell bodies (i.e. outside the ring, Fig. 2F, open arrowheads), whereas cones at the rim of the ring project their long processes toward the center of the ring (i.e. inside the ring, Fig. 2G, H, arrowheads). In addition, COS appeared outside the rings (Fig. 2H, arrows).

Vertical sections of normal retina that were labeled immunologically by PKC α showed apical processes directed toward the ONL (Fig. 2C). In RP retina, rod bipolar cells showed retracted processes (Fig. 2I, asterisk) or laterally distributed dendritic processes of rod bipolar cells in the OPL (Fig. 2I, arrows). In addition we observed misplaced rod bipolar cells at the outer part of the retina (proximal of OPL, Fig. 2I, arrowhead). In the whole-mount retina, we observed homogeneously distributed cell bodies of rod bipolar cells and their dendritic processes at the OPL in the normal retina (Fig. 2D). In RP retina, homogeneously distributed cell bodies and unevenly distributed dendritic processes of rod bipolar cells were observed at the level of OPL (Fig. 2J). Within the focal plane towards the outer part of the retina, we observed clustering of elongated-processes of rod bipolar cells (Fig. 2K, L) that were not clearly apparent in the whole-mount retina at the level of OPL (Fig. 2J). ON-bipolar cells immunologically labeled by Go α showed similar morphology to rod bipolar cells in vertical sections (Fig. 3A, E) and in whole-mounts (Fig. 3B, F-H) of normal and RP retinas. Therefore, our results indicate that dendritic processes of ON-bipolar cells, which include rod bipolar cells, undergo remodeling during spatial rearrangement of cones into ring mosaics in S334ter-line-3 retina.

Calbindin was used to view the entire horizontal cell and its plexus at the OPL (Fig. 3C, D, I-L). At P60 RP retina, the processes of horizontal cells were deteriorated (Fig. 3I, arrows) and cell bodies were misplaced (Fig. 3I, arrowhead) compared to that of P60 normal retina (Fig. 3C). In the RP whole-mount retina, homogenously distributed cell bodies with a somewhat discontinuous plexus were observed at the level of OPL (Fig. 3J). Within the focal plan towards the outer part of the retina, we observed clear cell bodies and their processes (Fig. 3K, L) that were not clearly apparent in the whole-mount RP retina at the level of OPL (Fig. 3J). Although we did not observe obvious changes in the processes of horizontal cells as in bipolar cells (i.e. clustering of elongated-processes), these horizontal cells formed a loose plexus compared to horizontal cells of the normal retina (Fig. 3D). This is consistent with the findings in RP vertical section showing deteriorated processes of horizontal cells at the OPL (Fig. 3I).

3.2. Presence of synaptic organization in remodeled cones in RP

To determine if remodeled cones maintain their synaptic organization, first we performed immunohistochemical double labeling experiments using antisera against M-opsin with synaptophysin or bassoon in the vertical sections of normal (Fig. 4A, C) and RP (Fig. 4B, D) retinas. Synaptophysin is an integral membrane protein of the synaptic vesicles. It plays an important role in synaptic vesicle formation and exocytosis in neurotransmitter delivery (Brandstatter et al. 1996). Bassoon is a presynaptic protein located at the synaptic ribbon in photoreceptor terminals in the OPL (Brandstatter et al. 1999). Both are markers for synaptic function in the photoreceptor. In vertical section of normal retina, axon terminals of M-opsin cones were heavily immunoreactively labeled with synaptophysin (Fig. 4A) and bassoon (Fig. 4C) at the OPL. In RP retina, synaptophysin (Fig. 4B) and bassoon (Fig. 4D) were remarkably decreased and localized at axons (Fig. 4B, arrowhead) and axon terminals (Fig.

4B and D, arrows) of cones. In whole-mount retinas, two different optical levels were assessed to examine the spatial distribution of M-opsin cones with synaptophysin (Fig. 5A-F) or bassoon (Fig. 5G-L). At the outer part of the retina where processes of cones project towards inside the ring (Fig. 5A, G), synaptophysin and bassoon were concentrated inside of the ring (Fig. 5B, H). At the optical level closer to OPL, M-opsin immunoreactivity was found at the processes of cones outside the ring (Fig. 5D, J). Synaptophysin and bassoon immunoreactivities were more concentrated outside the ring than at inside the ring (Fig. 5E, K). Merged images of M-opsin and synaptophysin or bassoon indicated that remodeled cones are able to maintain their synaptic integrity at the different depths of the outer retina (Fig. 5C, F, I and L).

3.3. Synaptic connections between second-order neurons and remodeled cones in rings

To determine if rearranged processes of second-order neurons make synaptic contact with M-opsin cones, we performed double immunohistochemical labeling using antisera against PKC α with synaptophysin or bassoon in the whole-mount retinas (Fig. 6). Whole-mount pictures were taken at two different optical levels, as per Figure 5. At the outer part of the retina, clustering of long dendritic processes of rod bipolar cells was observed (Fig. 6A, G). These elongated processes made contact with synaptophysin (Fig. 6C) and bassoon (Fig. 6I) inside the ring at the outer retina. At the level of the OPL, dendritic processes of rod bipolar cell outside the ring (Fig. 6D, J) made closer contact with synaptophysin (Fig. 6F) and bassoon (Fig. 6L). We also observed similar results with Go α and synaptic proteins in the RP whole-mount retina (data not shown). For the double labeling of horizontal cells with synaptophysin and bassoon, we observed that synaptophysin and bassoon were closely associated with deteriorated processes (Fig. 7, arrow) of horizontal cells in the vertical

sections of the RP retina. These data suggest that the dendritic processes of bipolar cells and horizontal cells maintain their synaptic connections with remodeled cones in rings.

3.4. Effect of TIMP-1 on the dendritic processes of second-order neuron and their connections to cones

In our recent study (Ji et al. 2015), we discovered that exogenous application of TIMP-1 significantly modulates the mosaics and restores its homogeneity without damaging the cones in the RP retinas (Fig. 1C). We further investigated the impact of TIMP-1 on the spatial distribution of second-order neurons and their connection with cones in the RP retina. In the TIMP-1 treated RP whole-mounts, M-opsin cones were homogenously distributed and their processes showed more homogeneous spatial pattern at the OPL (Fig. 8A, D). Synaptophysin (Fig. 8B) and bassoon (Fig. 8E) were localized at processes of M-opsin cones (Fig. 8C, F). TIMP-1 also disrupted the clustering of elongated-processes of rod bipolar cells at the OPL (Fig. 8G, J). Double immunohistochemistry with PKC α (Fig. 8G, J) and synaptophysin (Fig. 8H) or bassoon (Fig. 8K) showed connection between dendritic processes of rod bipolar cells and synaptic proteins (Fig. 8I, L). These results indicate that rod bipolar cells maintain their synaptic connections with M-opsin cones even after the cones have regained homogeneously distribution with TIMP-1.

To further investigate the synaptic connection between M-opsin cones and bipolar cells, we conducted triple immunohistochemistry experiments using antisera against M-opsin, Go α and VGLUT-1. VGLUT is a protein which loads glutamate into synaptic vesicles and participates in glutamate synaptic transmission in the neuron. Among the three types of VGLUT (VGLUT-1, VGLUT-2, and VGLUT-3), VGLUT-1 is the major isoform in the retina (Sherry et al. 2003). It is known to co-localize with synaptophysin at the OPL of the normal rat retina (Gong et al. 2006). We also observed co-localization of VGLUT-1 and

synaptophysin at the OPL in the RP retina (Fig. 9A-C). In RP whole-mount retina, two different optical levels were again probed to determine the synaptic connection between M-opsin cones and ON-bipolar cells. Triple immunological stain demonstrated synaptic connection between the dendritic processes of ON-bipolar cells and the processes of cones inside the ring at the outer part of the retinas (Fig. 9D). At the level of the OPL, dendritic processes of ON-bipolar cells outside the ring made synaptic contact with cones (Fig. 9E). In the TIMP-1 treated RP whole-mount retina, processes of M-opsin cones showed more homogeneous spatial pattern at the OPL (Fig. 8A, D). In parallel with this observations, processes of bipolar cells also rearranged to make connection with cones (Fig. 9F). Therefore, despite an extreme rearrangement of cone and bipolar cell processes, these cells maintain their synaptic connections before and after TIMP-1 treatment.

3.5. TIMP-1 protects the outer segments of the cones in RP

Previously, we have shown that exogenous application of TIMP-1 significantly modulates the mosaics without reducing the number of cell bodies of cones in the RP retinas (Ji et al. 2015). In the current experiments, we examined if TIMP-1 has an impact on COS by assessing the ratio between numbers of COS and cell bodies of M-opsin cones within the 1x1 mm² retinal areas (see methods) (Fig. 10). The M-opsin cones were labeled in the whole-mount retinas in P60 (Fig. 10A, C) and P90 (Fig. 10B, D) RP and TIMP-1 treated RP retinas. In P60 and P90 RP retinas, we observed ring-like patterns of M-opsin cones. By contrast, in TIMP-1 treated RP retinas, cones rearranged themselves into a more homogeneous pattern. At higher magnification of P90 whole-mount retinas, M-opsin cones were devoid of COS in RP retina (Fig. 10E, arrow), whereas most M-opsin cones maintained their COS at the same age in TIMP-1 treated retina (Fig. 10F). In P60 RP retinas, the mean total numbers of cell bodies and COS of M-opsin cones were $3,960 \pm 310$ cells/mm² and $2,512 \pm 49$ COS/mm²,

respectively. In P60 TIMP-1-treated RP retinas, the mean total numbers of cell bodies and COS of M-opsin cones were $4,477 \pm 760$ cells/mm² and $3,335 \pm 142$ COS/mm², respectively. The mean ratio of COS to cell bodies was 0.637 in P60 RP and 0.757 in P60 TIMP-1-treated RP retina. In P90 RP retinas, the mean total numbers of cell bodies and COS of M-opsin cones were $2,709 \pm 388$ cells/mm² and $1,499 \pm 63$ COS/mm², respectively. In P90 TIMP-1 treated RP retinas, the mean total numbers of cell bodies and outer segments were $3,253 \pm 91$ cells/mm² and $2,632 \pm 133$ COS/mm², respectively. The mean ratio of COS to cell bodies was 0.585 in P90 RP and 0.809 in P90 TIMP-1 treated RP retinas. The ratio of P90 TIMP-1 treated RP retina was statistically significantly different from the ratio of P90 RP retinas ($p=0.0019$, two-way ANOVA), while it was not significantly different from P60 TIMP-1 treated retina (Fig. 10G). Together, these results verify that TIMP-1 treatment prevents cone outer segment degeneration in RP retina.

4. Discussion

4.1. Spatial distribution of dendritic processes of second-order neurons and their synaptic connections to cones

In the present study, we observed retracted processes or laterally distributed processes of ON-bipolar cells (including rod bipolar cells) at the retinal OPL (Fig. 2, 3). These are in addition to misplaced bipolar cells at the outer part of the RP retina as described by others (Strettoi et al. 2003; Cuenca et al. 2004; Ray et al. 2010). Our previous work on RP rhodopsin S334ter-line-3 rat documented the loss of rods and the induction of a slow rearrangement of cones into regular mosaics of rings (Lee et al. 2011; Ji et al. 2012; Zhu et al. 2013). To determine the consequences of the cone migration on the second-order neurons, we evaluated the spatial distribution of second-order neurons and their dendritic processes in RP whole-mount retina (Fig. 2, 3). In the whole-mount, we found homogeneously distributed cell

bodies of ON-bipolar cells and their dendritic processes at the OPL level (Fig. 3F). At the focal plane towards the outer part of the retina, we identified a clustering form of elongated-dendritic processes of bipolar cells (Fig. 3H), which changes in bipolar cells and may reflect a compensatory response to maintain their synapses with the cone migration. Indeed, we observed remodeled dendritic processes of bipolar cells making synaptic contact with processes of cones inside and outside the ring (Figs. 4, 5, 6). Our results clearly show a spatial distribution of synaptophysin and bassoon at different depths of the outer retina and support the concept that remodeled cones are still maintaining their synaptic connections with bipolar cells (Fig. 5). The dendritic processes of horizontal cells show fewer obvious changes as in bipolar cells; however, they formed a loose plexus and still made connections with cones in the outer retina (Fig. 7). These results are consistent with previous reports showing ectopic synapses between existing cones and second-order neurons in the vertical section of other retinal degenerative animal models (Cuenca et al. 2004; Martinez-Navarrete et al. 2011). Despite the spatial changes in cones in RP retina, cones still maintain their synaptic connections with bipolar cells and continue to send the appropriate downstream pathway signals to RGCs. This is supported by optokinetic responses (McGill et al. 2012) and extracellular recordings of RGCs (Yu WQ, et al. IOVS 2014; 55: ARVO E-Abstract 6198) (Sekirnjak et al. 2011). Moreover, we have found that the shape of the receptive field of RGCs change in RP retina. The structure is reminiscent of that observed in the cone mosaic. In addition, when the receptive fields of individual RGC were summed together, a spatial sensitivity map across the retina revealed the ring-like pattern exhibited by COS in RP retina (Yu WQ, et al. IOVS 2014; 55: ARVO E-Abstract 6198).

4.2. Homogenously distributed M-opsin cones maintain synaptic connections with second-order neurons

TIMP-1 treatment led to a significant disruption of the M-opsin cone ring in RP retinas and resulted in almost complete restoration of homogeneous mosaics (Ji et al. 2015). In this study, we showed that TIMP-1 treatment rearranged cones and promoted the return of dendritic processes of second-order neuron to a homogeneous spatial pattern. Moreover, even with this dramatic event, the synapses between cones and second-order neurons were well preserved (Fig. 9). However, it is unclear if cones co-migrated with dendrites from the original second-order neurons or cone migration requires detachment from the dendrites and reformation of new synapses after relocation to the new position. Consistent with the former hypothesis, we observed rod bipolar cells with extended dendritic processes of bipolar cells inside the cone rings in RP retina (Fig. 2). Several groups have demonstrated that second-order neurons adapt the length of their dendritic processes with changes in location of photoreceptor terminals in the ONL (Fisher et al. 2005; Sullivan et al. 2007; Ray et al. 2009). Therefore, bipolar cells could have retracted their dendrites when cones were redistributed by TIMP-1. Alternatively, sprouts from retinal neurons are commonly visualized in RP models (Strettoi et al. 2002; Cuenca et al. 2004; Cuenca et al. 2005; Garcia-Ayuso et al. 2013) and Dick and colleagues have demonstrated that new dendrites from rod bipolar cells formed ectopic synapses with cones in a bassoon mutant mouse (Dick et al. 2003). Thus, during TIMP-1 treatment, cones could have formed connections with new bipolar cells at the new location.

Can TIMP-1 alone alter the length of axons or dendrites of retinal neurons? It is possible that TIMP-1 reduced the length of axons and dendrites to affect this rearrangement between cones and second-order neurons. This hypothesis is supported by published data that TIMP-1 is involved in modulating neurite length in cortical neurons (Jourquin et al. 2005; Ould-yahoui et al. 2009). Overall, the elimination of ring mosaic of cones by TIMP-1 while

retaining synaptic connections between cones and second-order neurons could help to mitigate some of the consequences of cone rings on RGC functions.

4.3. Preventive effect of TIMP-1 on COS degeneration

In our previous studies, we have neither observed significant differences in the total number of M-opsin cones nor observed differences in their cell body densities in P180 RP retinas (Ji et al. 2012). However, our previous and current experiments reveal a distorted or reduced loss of COS in late stages of RP (Fig. 10) (Ji et al. 2012). We found that TIMP-1 increases the mean ratio of COS to cell body at P90 compared to the ratio in control RP retina (Fig. 10G). These data suggest that TIMP-1 prevents the degeneration of COS in RP retina (Fig. 10). TIMP-1 is an inhibitor of matrix metalloproteinases (MMPs), specifically MMP-9, and functions to maintain ECM homeostasis (Murphy et al. 2003). In retinal excite-toxicity models in rodents, a specific MMP-9 inhibitor, or agents acting on downstream targets in the MMP-9 pathway prevented pathological remodeling of the inner limiting membrane and detachment-induced cell death of RGCs (Zhang and Chintala 2004; Manabe et al. 2005; Santos et al. 2012). Furthermore, the increase in retinal TIMP-1 demonstrated in the glaucoma model is likely to facilitate its neuroprotective effect on RGCs through both inhibition of MMP-9 and anti-apoptotic actions. Additionally, recent studies have shown that TIMP-1 has neuroprotective effects that are independent of its ability to inhibit MMPs (Tan et al. 2003). Therefore, exogenous application of TIMP-1 in RP retina may serve to counter balance the simultaneous increase in MMP-9 to protect COS or it may protect COS independently of MMP. However, TIMP-1 did not prevent the shortening of COS in RP retina (Fig. 10), which may be due, in part, to the timing of the TIMP-1 treatment in RP. Earlier treatment of TIMP-1 before shortening of COS may have a significant impact on COS survival in RP. Based on these current studies, we are now examining the cellular pathways

driving cone survival and the expression levels of MMP-9 in RP retina with or without TIMP-1 treatment.

Acknowledgments

We thank Dr. Matthew M. LaVail for kindly providing us with animals. The work was supported by a VSoE Research Innovation Fund to E-JL, and by National Science Foundation Grant 0310723 and National Eye Institute Grants EY016093 and EY11170 to NMG, and EY015851 to CMC, CMC is the inaugural Mary D. Allen Endowed Chair in Vision Research (Doheny Eye Institute), NEI core grant: EY03040 (Doheny Eye Institute), Research to Prevent Blindness (USC Ophthalmology), the Mary D. Allen Foundation (CMC) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A6A3A03059828)

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Figure Legends

Figure 1. Confocal micrographs of whole-mounts processed for M-opsin immunohistochemical staining in normal rat (A), saline injected RP retina (B), and TIMP-1 injected RP retina (C) at P60. Saline and TIMP-1 (25 $\mu\text{g/ml}$) are injected at P45. Cones in TIMP-1 treated RP retinas (C) have rearranged themselves into a homogeneous pattern, similar to normal retina (A), while cones in RP retinas showed ring-like pattern. P, postnatal; N, Normal; RP, Retinitis Pigmentosa; TIMP-1, Tissue Inhibitor of Metalloproteinase-1, Scale bar = 100 μm

Figure 2. Confocal micrographs of vertical sections (A, C, E and I) or whole-mounts (B, D, F-H and J-L) labeled with M-opsin (A, B, E-H) and PKC α (C, D, I-L) in normal (A-D) and RP (E-L) retinas at P60. Pictures for whole-mounts were taken in different optical levels (refer to dotted lines in vertical sections). In normal retina, M-opsin cones were vertically aligned (A) and homogeneously distributed (B). Rod bipolar cells were labeled with PKC α (C). Cell bodies and dendritic processes of rod bipolar cells were homogeneously distributed (D). In RP retina, M-opsin labeled cones showed separate regions full of clusters of cell bodies and, outer segment (E, arrow) and processes (E, open arrowhead) followed by regions devoid of cell bodies but richer in long processes (E, arrowhead) in the vertical section. In whole-mounts, M-opsin cones that were distributed outside the ring project their processes close to their cell bodies at the OPL level (F, open arrowheads) whereas cones at the rim of the ring project their long processes toward the inside the ring at the outer part of the retina (G, H, arrowheads). Cone outer segments appeared outside the rings (H, arrows). PKC α labeled rod bipolar cells showed retracted processes (I, asterisk), laterally distributed

dendritic processes (I, arrows) and misplaced cell body at the outer part of the retina (I, arrowhead). Bipolar cells showed clustering of elongated-processes towards the outer part of the retina (J-L) in RP retina. RP, Retinitis Pigmentosa; PR, photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; PKC α , protein kinase C α . Scale bar = 25 μ m in A, C and 50 μ m in B, D, E-L.

Figure 3. Confocal micrographs of vertical sections (A, C, E and I) or whole-mounts (B, D, F-H and J-L) labeled with Go α (A, B, E-H) and Calbindin (C, D, I-L) in normal (A-D) and RP (E-L) retina at P60. Pictures for whole-mounts were taken in different optical levels (refer to dotted lines in vertical sections). In normal retina, Go α labeled ON bipolar cells and calbindin labeled horizontal cells showed homogeneously distributed cell bodies and processes (A-D). In RP retina, ON bipolar cells showed retracted processes (E, asterisk), laterally distributed dendritic processes (E, arrows) and misplaced cell body at the outer part of the retina (E, arrowhead) in the vertical section. Horizontal cells showed deteriorated processes (I, arrows) and misplaced cell bodies (I, arrowhead). In whole-mounts, ON bipolar cells showed clustering of elongated-processes towards the outer part of the retina (F-H) and horizontal cells showed loose plexus (J-L) in RP retina. RP, Retinitis Pigmentosa; PR, photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 25 μ m in A, C and 50 μ m in B, D, E-L.

Figure 4. Confocal micrographs of vertical sections double-labeled with M-opsin (red) and either synaptophysin (green, A, B) or bassoon (green, C, D) in normal retina (A, C) and RP

retina (B, D) at P60. Synaptophysin (B) and bassoon (D) were decreased and localized at axons (B, arrowhead) and axon terminals (B, D, arrows) of cones. N, Normal; RP, Retinitis Pigmentosa; PKC α , protein kinase C α ; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar = 20 μ m

Figure 5. Confocal micrographs of whole-mounts double-labeled with M-opsin (red) and either synaptophysin (green, B, C, E and F) or bassoon (green, H, I, K and L) in RP retina at P60. Images were taken at two different optical levels; at the outer part of retina (A-C and G-I) and near OPL level (D-F and J-L). Saline is injected at P45. Synaptophysin (B) and bassoon (H) were concentrated inside the ring and co-localized with long processes of M-cones (C, I) at the outer part of the retina. Synaptophysin (E) and bassoon (K) were more concentrated outside the ring at the optical level closer to OPL, and co-localized with M-cone processes (F, L). RP, Retinitis Pigmentosa; postnatal day, P SYN, synaptophysin; PKC α , protein kinase C α ; OPL, outer plexiform layer. Scale bar = 50 μ m

Figure 6. Confocal micrographs of whole-mounts double-labeled with PKC α (red) and either synaptophysin (green, B, C, E and F) or bassoon (green, H, I, K and L) in RP retina at P60. Images were taken at two different optical level; at the outer part of retina (A-C and G-I) and near OPL level (D-F and J-L). Saline is injected at P45. Synaptophysin (B) and bassoon (H) were concentrated inside the ring and made contact with elongated dendritic processes of rod bipolar cell (C, I) at the outer part of the retina. Synaptophysin (E) and bassoon (K) were more concentrated outside the ring at the optical level closer to OPL, and contact with dendritic processes of rod bipolar cell (F, L). RP, Retinitis Pigmentosa; SYN, synaptophysin; PKC α , protein kinase C α ; OPL, outer plexiform layer. Scale bar = 50 μ m

Figure 7. Confocal micrographs of vertical sections immunologically double-labeled with calbindin (red) and either synaptophysin (green, A, B) or bassoon (green, C, D) in normal retina (A, C) and RP retina (B, D) at P60. Synaptophysin and bassoon made contact (arrows) with deteriorated processes of horizontal cells. N, Normal; RP, Retinitis Pigmentosa; PKC α , protein kinase C α ; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar = 20 μ m

Figure 8. Confocal micrographs of whole-mounts double-labeled with M-opsin (red, A, C, D and F) or PKC α (red, G, I, J and L), and either synaptophysin (green, B, C, H and I) or bassoon (green, E, F, K and L) in TIMP-1 treated RP retina at P60. TIMP-1 is injected at P45. Synaptophysin (B) and bassoon (E) were homogeneously distributed at the OPL and co-localized with processes of M-opsin cones (C, F). Synaptophysin (H) and bassoon (K) staining also showed connection between dendritic processes of rod bipolar cells (I, L). RP, Retinitis Pigmentosa; TIMP-1, Tissue Inhibitor of Metalloproteinase-1; SYN, synaptophysin; PKC α , protein kinase C α . Scale bar = 50 μ m

Figure 9. Confocal micrographs of vertical sections immunologically double-labeled with VGLUT-1 (red, A, C) and synaptophysin (green, B, C) in RP retina, and whole-mounts immunologically triple-labeled with M-opsin (green), Go α (blue) and VGLUT-1 (red) in saline-injected RP retina (D and E) and TIMP-1 injected RP retina (F) at P60. VGLUT1 and synaptophysin were co-localized in RP retina (C). At the outer part of the retina, clustering form of elongated-processes of ON-bipolar cells made synaptic contact with the processes of cones inside the ring (D). At the level of the OPL, dendritic processes of ON-bipolar cells

made synaptic contact with cones outside the ring (E). In the TIMP-1 treated RP retina, processes of M-cones and bipolar cells rearranged and maintain their connection at the OPL (F). RP, Retinitis Pigmentosa; VGLUT-1, vesicular glutamate transporter 1; TIMP-1, Tissue Inhibitor of Metalloproteinase-1; P, postnatal days; PR, photoreceptor; OPL, outer plexiform layer. Scale bar = 5 μ m in A-C, 50 μ m in D-F and 10 μ m in inset.

Figure 10. Confocal micrographs of whole-mounts processed for M-opsin immunohistochemical staining in saline injected RP retina (A, C and E) and TIMP-1 injected RP retina (B, D and F) at P60 (A and B) and P90 (C-E and F). Saline and TIMP-1 are injected at P45. Histogram showed the ratio of number of cone outer segment (COS) per cell body (G). The COS per cell body ratio of P90 TIMP-1 treated RP retina was significantly higher than the ratio of P90 RP retinas ($p=0.0019$, G). RP, Retinitis Pigmentosa; TIMP-1, Tissue Inhibitor of Metalloproteinase-1; P, postnatal day. Scale bar = 100 μ m. The symbol * indicates $p < 0.05$

Figure 1

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