Immunohistochemical Evaluation of Idiopathic Epiretinal Membranes and In Vitro Studies on the Effect of TGF-β on Müller Cells

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PURPOSE. The purpose of this study was to investigate the presence of type VI collagen and glial cells in idiopathic epiretinal membrane (iERM) and the role of TGF-β in the expression of collagens and α-smooth muscle actin (α-SMA) in retinal Müller cells.

METHODS. Idiopathic ERM samples from vitrectomy were analyzed for glial acidic fibrillary protein (GFAP), cellular retinaldehyde-binding protein (CRALBP), α-SMA, and type VI collagen using flat-mount immunohistochemistry. To study intracellular collagen expression in relation to cellular phenotype, spontaneously immortalized human Müller cells (MIO-M1) were treated with TGF-β1 for 48 hours, and the expression of α-SMA and intracellular type I, II, IV, and VI collagens was studied by using immunocytology. Findings in Müller cells were compared with those in fetal lung fibroblasts and newborn skin fibroblasts.

RESULTS. A colocalization of GFAP/CRALBP and GFAP/α-SMA was found in iERM, indicating a dynamic process of activation of retinal Müller cells in vivo. Transforming growth factor-β1 induced up-regulation of α-SMA stress fibers in retinal Müller cells and both types of fibroblasts in vitro. The intracellular staining intensity of type I, II, and VI collagens was decreased in retinal Müller cells containing α-SMA stress fibers, whereas the intracellular staining intensity of type I and VI collagens in both types of fibroblasts was not affected.

CONCLUSIONS. Type VI collagen and activated retinal Müller cells are present in iERM. Transforming growth factor-β1 induces an up-regulation of α-SMA stress fibers in retinal Müller cells and fibroblasts and appears to have a cell-specific effect on intracellular collagen expression.

Keywords: retinal Müller cells, collagen, TGF-β, idiopathic epiretinal membranes
the proliferation, migration, and transdifferentiation of retinal involved in the formation of epiretinal membrane by regulating collagens in fibroblasts was seen.\textsuperscript{27,28} Therefore, we hypothesized that type VI collagen is one of the ECM proteins involved in the dynamic turnover of the vitreo-retinal matrix in physiologic and pathologic situations.\textsuperscript{26} Therefore, we hypothesized that type VI collagen is one of the ECM proteins in the iERM and that TGF-\(\beta\) stimulates the retinal Müller cells to transdifferentiate into a myofibroblast-like phenotype that is responsible for the up-regulation of type VI collagen. This hypothesis is based on previous studies in which a TGF-\(\beta\)-induced up-regulation of the expression of type I and VI collagens in fibroblasts was seen.\textsuperscript{27,28} The present study evaluates flat-mounted surgical specimens of iERM for evidence of the presence of type VI collagen and of transdifferentiation of retinal Müller cells, that is, cells expressing both \(\alpha\)-SMA (myofibroblast marker) and glial acidic fibrillary protein (GFAP; marker of Müller cells and Müller cell activation). In addition, we compared the staining patterns of collagens in cultured TGF-\(\beta\)-treated retinal Müller cells with those in cultured TGF-\(\beta\)-treated newborn skin and fetal lung fibroblasts.

**MATERIALS AND METHODS**

**Inner Limiting Membrane/ERM Flat Mount and Immunofluorescence**

**General Procedures.** Twenty-two patients (16 females and 6 males) who were referred to the Ophthalmology Department of the University Medical Center Groningen for iERM surgery were involved in the study between June 2010 and June 2012. The mean age of the patients was 69.6 years (range, \(53-84\) years). Prior to their scheduled surgery, a complete ophthalmic examination was performed, and written informed consent was obtained. This study was conducted in accord with the Declaration of Helsinki and was approved by the Institutional Review Board of the University Medical Center Groningen.

The surgical technique involved a standard three-ports pars plana vitrectomy, creation of a posterior vitreous detachment if necessary, and inner limiting membrane (ILM)/ERM peeling with the aid of Membrane blue (DORC, Zuidland, The Netherlands). Peeling was performed with an end-grIPPING forceps, intending to remove the ERM and ILM over an area of more than 1.5 disc diameters surrounding the macula. All vitrectomy procedures were performed by two experienced vitreo-retinal surgeons (coauthors VWRL and GP). The ERM/ILM samples were immediately processed for flat-mount immunohistochemical analysis.

**Flat-Mount and Immunohistochemistry of ILM/ERM Specimens.** The procedures for flat-mount and immunohistochemistry of ILM/ERM specimens were described previously.\textsuperscript{29} Briefly, the ILM/ERM specimens were placed on a silicone elastomer (SYLVARD 184; Dow Corning Corporation, Midland, MI, USA)–coated petri dish in 200 \(\mu\)L 1% PBS containing 2% Tween 20 (PBST; Bio-Rad, Hercules, CA, USA). Using a stereomicroscope, the specimens were flattened with fine glass sticks and pinned to the dish with stainless steel pins (Austerlitz Insect Pins; Fine Science Tools, Inc., Foster City, CA, USA). Subsequently, the flattened specimens were fixed in 2% paraformaldehyde for 30 minutes, washed three times in PBST for 15 minutes, blocked with PBST containing 5% BSA (Sigma-Aldrich Corp., St. Louis, MO, USA) for 60 minutes, and incubated overnight at 4°C in a mixture of two primary antibodies (both diluted to 1:200). Primary antibodies used included rabbit (polyclonal; Abcam, Cambridge, UK) or mouse (monoclonal; Sigma-Aldrich Corp.) anti-GFAP (glial cell marker, may be present in quiescent Müller cells, but is up-regulated in case of Müller cell activation); rabbit anti-cellular retinaldehyde-binding protein antibody (CRALBP; Müller cell marker, UW55, a kind gift from JC Saari, University of Washington, Seattle, WA, USA), mouse anti–\(\alpha\)-SMA (monoclonal; Sigma-Aldrich Corp.), and rabbit anti–type VI collagen antibody (polyclonal; Abcam). The combinations of the primary antibodies used in the study included the following: CRALBP/GFAP (2 cases), CRALBP/\(\alpha\)-SMA (2 cases), GFAP/\(\alpha\)-SMA (4 cases), CRALBP/\(\alpha\)-SMA/Type VI collagen antibodies (2 cases), and type VI collagen/\(\alpha\)-SMA (2 cases). Two samples were used as negative controls for the primary antibodies.

The samples were rinsed three times with PBST for 15 minutes and incubated for 4 hours at 4°C with two fluorescent-labeled secondary antibodies (diluted 1:200) combined with 4′-diamino-2-phenylindole (DAPI; diluted 1:200 for visualization nuclei; Sigma-Aldrich Corp.). Secondary antibodies used include donkey anti-rabbit antibody conjugated with RedX, donkey anti-goat antibody with FITC, and donkey anti-mouse with FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

After incubation, the specimens were washed three times in PBST for 15 minutes and removed from the petri dish. Using a stereomicroscope, the specimens were flattened on a glass slide in a drop of antifadent (AF1; Citifluor, Ltd., London, UK) and sealed with a cover slip. Negative control samples underwent the entire procedure, except for the application of the primary antibodies. To confirm the specificity of the anti-collagen I, II, IV, and VI antibodies, labeling procedures were done on samples from a paraffin-embedded human donor eye containing vitreous body, retina, choroid, and sclera. Control samples consisted of sections that underwent the entire procedure, without application of the primary antibody and sections that were incubated with an isotype control goat or rabbit IgG (1:150; Southern Biotechnology Associates [SBA], Birmingham, AL, USA) and underwent the further labeling procedure.

**Effect of TGF-\(\beta\) on Intracellular Collagen Expression in \(\alpha\)-SMA–Positive Cells**

**Cell Culture.** The spontaneously immortalized human Müller cell line MIO-MI (a kind gift from GA Limb, UCL Institute of Ophthalmology, London, UK) has been confirmed to contain important characteristics of human Müller cells, such as the expression of CRALBP and only limited expression of GFAP.\textsuperscript{30} Dulbecco’s modified Eagle’s medium with high glucose containing \(\gamma\)-glutamax I (Life Technologies, Inc., Rockville, MD, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Life Technologies, Inc.) was the stock medium. The stock culture of MIO-MI
(passage between 61 and 70) was maintained in stock medium at 37°C with 5% CO₂ for the experiments.

Fetal lung fibroblasts were incubated in DMEM with high glucose containing t-glutamax I (Life Technologies, Inc.) supplemented with gentamicin/glutamin and 10% FBS at 37°C with 5% CO₂.

Newborn skin fibroblasts (CCD-1112Sk; American Type Culture Collection, Manassas, VA, USA) were incubated in stock medium at 37°C with 5% CO₂.

Effect of TGF-β1 on Retinal Müller Cells. The cells were seeded on glass cover slips in six-well plates in their stock culture medium with the addition of 0.2 mM ascorbic acid. After 24 hours of incubation, 10 ng/mL recombinant TGF-β1 was added to the medium for another 48 hours of culture. Subsequently, the cells were processed for immuno-double labeling to identify the colocalization of α-SMA and collagens including type I, II, IV, and VI collagens. The primary and secondary antibodies used are listed in Table 1. Briefly, the cells were washed three times in PBS and fixed in 1:1 acetone/methanol at −20°C for 10 minutes. Then, the fixative was discarded, and the slides were air-dried and stored at −20°C until further use. After blocking with 5% BSA (Sigma-Aldrich Corp.) in PBS, the cells were incubated in a mixture of two secondary antibodies according to the host of the primary antibodies and DAPI as described previously for 2 hours in a dark room. After washing in PBS, a drop of antifade (AF1; Citifluor, Ltd., London, UK) was applied to each cover slip, and the cover slips were placed on a slide and sealed. For negative controls, the samples underwent the entire procedure, and the primary antibody was omitted.

Western blot analysis was used to check the specificity of the antibodies and to evaluate intracellular protein expression (GFAP, α-SMA, and type VI collagen). For this, MIO-M1 cells were harvested in denaturation buffer (10 mM Tris-HCl, pH 7, containing 1 mM EDTA, 2.5% SDS, 5% 2-mercaptoethanol, and 10% glycerol), and proteins were analyzed by SDS-PAGE according the method of Laemmli using a 7.5% running gel as previously described.26 After separation, the gel was blotted to nitrocellulose and blocked for 1 hour with 2% skimmed milk. After incubation overnight with the primary antibody, the secondary antibody was added and allowed to incubate for 1 hour (Table 1). Then, the blot was incubated with alkaline phosphatase (AP)-conjugated tertiary antibody diluted 1:250 for another hour. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in AP buffer. All incubation and washing steps were performed at room temperature. The primary antibodies rabbit anti-collagen I and VI (Table 1) were incubated in mouse anti rabbit IgG (Jackson Immunoresearch Laboratories) first and then in goat anti-mouse AP (Bio-Rad); goat anti-collagen IV (Table 1) was incubated in mouse anti-goat IgG (Jackson Immunoresearch Laboratories, Inc.) first and then in goat anti-mouse AP (Bio-Rad); goat anti-collagen IV (Table 1) was incubated in mouse anti-goat IgG (Jackson Immunoresearch Laboratories, Inc.) first and then in goat anti-mouse AP (Bio-Rad). The ratio between the protein of interest and the loading standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam) was calculated using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Table 1. Antibodies

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<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
<th>Tertiary Antibodies</th>
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<td>Immunohistochemistry for iERM specimens</td>
<td>Rabbit anti-CRALBP (1:200; from JC Saari, University of Washington)</td>
<td>Red-X-conjugated donkey anti-rabbit (1:200; Jackson Immunoresearch Laboratories)</td>
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<tr>
<td></td>
<td>Rabbit anti-GFAP (1:200; Abcam)</td>
<td>FITC-conjugated donkey anti-rabbit (1:200; Jackson Immunoresearch Laboratories)</td>
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<tr>
<td></td>
<td>Mouse anti-α-SMA (1:200; Sigma-Aldrich Corp.)</td>
<td>FITC-conjugated donkey anti-mouse (1:200; Jackson Immunoresearch Laboratories)</td>
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<td>Immunocytochemistry for MIO-M1 cells and fibroblasts</td>
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<td>Red-X-conjugated donkey anti-mouse (1:200; Jackson Immunoresearch Laboratories)</td>
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<td></td>
<td>Rabbit anti-type I collagen (1:100; Abcam)</td>
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<td></td>
<td>Rabbit anti-type IV collagen (1:100; Abcam)</td>
<td>(1:200; Jackson Immunoresearch Laboratories)</td>
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<td></td>
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<td>FITC-conjugated donkey anti-goat (1:200; Jackson Immunoresearch Laboratories)</td>
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<td>Western blot</td>
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<td>Goat anti-mouse IgG (1:1000; Jackson Immunoresearch Laboratories)</td>
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<td>Mouse anti-α-SMA (1:500; Sigma-Aldrich Corp.)</td>
<td>Rabbit anti-goat/AP (1:250; Jackson Immunoresearch Laboratories)</td>
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</table>
RESULTS

Immunohistochemical Analysis of Flat-Mount Surgical Samples

**General Observations.** Positive immunostaining of anti-GFAP, anti-α-SMA, and anti-CRALBP was found in all tested samples (Table 2). Among the 15 samples with a positive GFAP signal, 10 showed a diffuse positivity throughout the entire cell membrane, whereas 5 contained small clusters of GFAP-positive cells (Fig. 1).

**Colocalization of GFAP/CRALBP and GFAP/α-SMA in iERM.** In iERM samples stained for GFAP/CRALBP, a coexpression of GFAP and CRALBP was observed in some of the cells, whereas other cells were only CRALBP positive (Fig. 2). These different staining patterns were in adjacent areas. Three of four iERM specimens stained for GFAP/α-SMA showed a coexpression of GFAP and α-SMA (Fig. 3).

**Presence of Type VI Collagen in iERM.** Nine of 10 (90%) iERM were positive for type VI collagen. In six of the immunopositive ERMs, type VI collagen formed a diffuse fine fibrillary network (Fig. 4). At the edge of the ERM, a direct attachment of the network to the underlying ILM could be visualized (Fig. 4B).

Immunocytologic Analysis of Cell Cultures

**TGF-β1–Induced Effects on MIO-M1 Cells.** The TGF-β1 treatment induced significant changes in MIO-M1 cells (Figs. 5E–H) including up-regulation of α-SMA stress fibers and down-regulation of the intracellular expression of type I, II, and VI collagens in MIO-M1 cells containing α-SMA-positive stress fibers.

<table>
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<tr>
<th>Antibodies</th>
<th>Number of Samples</th>
<th>Numbers of Positive Expression</th>
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<tr>
<td>GFAP</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>α-SMA</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>CRALBP</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Type VI collagen</td>
<td>10</td>
<td>9</td>
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</tbody>
</table>

The quiescent MIO-M1 cells exhibited intracellular positivity to type I, II, IV, and VI collagens (Figs. 5A–D, green). Extracellular deposition of type I, II, and VI collagens could be observed as irregular fibrillar structures. Also, a faint and diffuse cytoplasmic α-SMA positivity could be observed (Figs. 5A–D, red), but α-SMA stress fibers were rare. After 48 hours of incubation in 10 ng/mL TGF-β1, a significant up-regulation of α-SMA stress fibers was observed (Figs. 5E–H, red). Furthermore, the intracellular expression of type I, II, and VI collagens appeared to be decreased in MIO-M1 cells containing α-SMA stress fibers, whereas the cells that did not show α-SMA stress fibers maintained their intracellular expression of type I, II, and VI collagens (Figs. 5E–H). The expression of type IV collagen in α-SMA stress fiber–positive cells appeared to be the least affected. The staining of type IV collagen in α-SMA stress fiber–positive cells was less diffusely present than that in the cells without α-SMA–positive stress fibers and seemed to be more
concentrated around the nucleus. This may be caused by the fact that these cells are generally more spread, that is, occupy a larger surface area, than cells without α-SMA stress fibers.

By Western blot analysis, TGF-β1–stimulated Müller cell cultures showed an up-regulation of GFAP (3.5-fold increase), α-SMA (1.5-fold increase), and type VI collagen (27.7-fold increase) compared with unstimulated Müller cell cultures (Fig. 6).

**TGF-β1–Induced Effects on Fetal Lung Fibroblasts and Newborn Skin Fibroblasts.** The TGF-β1 treatment induced up-regulation of α-SMA stress fibers in both fetal lung fibroblasts and newborn skin fibroblasts. However, the intracellular staining of type I and VI collagens in both fibroblast cell lines containing α-SMA stress fibers was similar to that in the stress fiber–negative cells (Fig. 7).

**Specificity of the Antibodies.** By light microscopy, different labeling patterns were found for type I, II, IV, and VI collagens (Supplementary Fig. S1). Control samples in which the primary antibody was omitted and samples labeled with isotype controls (IgG) were negative.

By Western blot analysis, collagen bands could be appreciated at different heights for the different types of collagen, which is consistent with previous findings by our group, and confirms the specificity of the antibodies (Supplementary Fig. S2).

**DISCUSSION**

In this study, we identified type VI collagen as a component of iERMs. Also, we observed cells positive for GFAP and for α-SMA, which we consider to be transdifferentiated retinal Müller cells. In vitro, TGF-β appears to stimulate the retinal Müller cell to transdifferentiate into a myofibroblast-like phenotype expressing α-SMA stress fibers, at the same time decreasing intracellular collagen expression.

Consistent with previous studies of human Müller cells, MIO-M1 cells in response to TGF-β1 obtain an α-SMA stress fiber–expressing phenotype. Assuming that in vivo Müller cells will do the same, these cells will then be able to contract the ERM on which they are growing, which is corroborated by...
By being incorporated in the stress fibers, α-SMA is involved in the contractile activity of various cell types, such as fibroblasts, chondrocytes, and mesenchymal stem cells. The de novo expression of α-SMA alone has been reported to increase the contractile activity of fibroblasts. The progression of iERM can be considered as a fibrotic process because the pathologic findings are an increased collagen deposition and membrane contraction. Although some debate exists concerning the initial event of iERM, the general consent is that the retinal Müller cells are actively involved in the formation and contraction of iERM (Supplementary Fig. S3).

Cellular retinaldehyde-binding protein is known to be expressed in retinal Müller cells containing 11-cis-retinol and 11-cis-retinaldehyde, whereas the up-regulation of GFAP is a hallmark of retinal Müller cell activation. The simultaneous expression of CRALBP and GFAP can be used to differentiate Müller cells from hyalocytes that have also been shown to be responsible for the fibrotic tissue formation and expression of GFAP. In our clinicopathologic study, the coexpression of CRALBP/GFAP and α-SMA/GFAP in ERM samples indicates that the differentiated CRALBP-positive retinal Müller cell first becomes activated (GFAP positivity) and then is being...
transformed into a myofibroblast-like cell type (α-SMA positivity). Thus, the retinal Müller cell may actively be involved in the formation of iERM.

Furthermore, we frequently observed cells that only expressed CRALBP and not GFAP in the vicinity of these activated Müller cells. This indicates that the transdifferentiation process is both dynamic and focal. Local factors, yet to be identified, activate only the cells nearby, which then change their phenotype, become myofibroblast-like, and start to contract their matrix. Tissue stiffness is one of the important local factors that modulate the formation of myofibroblasts by activating TGF-β.

During the formation of iERM, retinal Müller cells proliferate and migrate to the vitreal surface of the retina, which is stiffer than the compliant retinal tissue. Because previous researches suggest that retinal Müller cells are mechanosensitive, it is possible that the increase in stiffness could provide a local environment that facilitates the transdifferentiation of retinal Müller cells to a myofibroblast-like phenotype.

In the current study, we show that the effect of TGF-β on retinal Müller cells was not exactly the same as that observed in fibroblasts. Our study suggests that TGF-β stimulation results in an overall increase in intracellular GFAP and type VI collagen and also some up-regulation of α-SMA. In those Müller cells that develop α-SMA stress fibers in response to TGF-β1, the intensity of intracellular staining for type I, II, and VI collagens seems to decrease. The TGF-β1 treatment did not affect the intracellular staining of type I and VI collagens in both types of fibroblasts. These findings indicate that when retinal Müller cells express α-SMA stress fibers, their synthesis of type I, II, and VI collagens is either down-regulated or their secretion of these collagens into the extracellular matrix is promoted. In fibroblasts of many organs, TGF-β1 has been well established to be responsible for excessive deposition of collagens during fibrotic tissue formation. Furthermore, TGF-β1 has also been shown to be involved in the regulation of procollagen processing and fibrillogenesis, which may result in promoting the secretion of collagens into the extracellular space. This suggests that TGF-β1, besides its potent activity to induce collagen production, can also promote the processing of procollagens to mature collagens. The possible suppression of intracellular type I, II, and VI collagen production may indicate a negative feedback mechanism that regulates the profibrogenic effect of TGF-β in stress fiber-positive retinal Müller cells. High doses of TGF-β have been shown to suppress the expression of type I collagen via the induction of a negative regulator of collagen transcription in renal fibroblasts.

Idiopathic ERMs contain a complex ECM protein network containing many types of collagens, including type I, II, III, IV, and VI collagens. Our observations suggest that type VI collagen forms a fine fibrillar network in iERM, which is consistent with previous reports.

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/Journals/IOVS/934564/)
reported the expression of type VI collagen predominantly in cellophane maculopathy as opposed to preretal macular fibrosis. In previous research, type VI collagen expression was not detected by light microscopy in normal human ILM at the macular region, nor in ERM from idiopathic macular hole patients with the same immunohistochemical technique. In this study, we used commercially available antibodies and negative and positive controls to reduce the chance of false interpretations due to antibody cross-reaction. Therefore, we conclude that the positive staining of type VI collagen is specific.

The use of a cell culture model has the advantage of offering a system in which the effects of individual variables such as TGF-β can be studied. Obvious limitations include the use of an in vitro system and isolated cells. Thus, findings will only serve to formulate hypotheses on the in vivo situation, but firm conclusions cannot be drawn. However, in the present study, in vitro findings and observations on surgically obtained IERMs seem to support each other.

In summary, our ex vivo and in vitro studies shed some new light on the pathogenesis of iERM regarding the involvement of retinal Müller cells and the production of type I, II, and VI collagens in response to TGF-β. Although certain debate exists concerning the initial events of iERM formation, retinal Müller cells are one of the important cell types that are actively involved by migration, proliferation, and transdifferentiation. During the process of iERM formation, TGF-β can be produced and activated by both retinal Müller cells and hyalocytes. In an autocrine or paracrine fashion, TGF-β up-regulates the expression of α-SMA in these epiretinal cells, resulting in the contraction of the epiretinal membrane. Although the retinal Müller cells that do not express α-SMA could continue their production of collagens to form the ECM network of the iERM, the α-SMA–expressing retinal Müller cells are responsible for the membrane contraction. Whether the reduced intracellular collagen staining in retinal Müller cells containing α-SMA stress fibers is due to reduced production or increased secretion is at present unresolved.

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